

nature

24 August 1978

## Counting astronomers in Europe

ASTRONOMERS sometimes like to count the number of objects in a portion of sky, and compare it with the numbers in other regions. They find the task beset with difficulties: how to define the objects, how to allow for changing obscurations in the different parts of the sky, and—most of all—how to draw conclusions from the results. This week the European Science Foundation publishes a survey of astronomers, comparing numbers and age distributions among the countries of Europe; and they seem to have come across much the same difficulties.

First there was a problem in defining "astronomers". In January 1977 the Astronomy Committee of the ESF sent out a circular asking member organisations for numbers and age distributions of "holders of PhDs or persons of equivalent competence working actively as scientists in a field of astronomy". The UK, for example, reporting through the UK's Science Research Council, included space scientists and aeronomers; other countries may have restricted themselves to the ground-based astronomers. Measurement techniques also varied, with ten of the reports coming through official bodies and six through individual astronomers. Different countries presented their data in different ways.

Furthermore the careers of astronomers vary greatly among ESF members. In Sweden, for example, a PhD is often not awarded until the student has done the equivalent of a few years' post-doctoral work in the UK. He or she is thus highly committed to astronomy, which may explain the Swedish experience that PhDs find great difficulty in obtaining jobs outside astronomy or teaching. There is even great variation among the Swedish universities; according to the ESF report 74% of PhDs from Stockholm, but only 18% from Uppsala remain in astronomy. Again, the UK, with 724 astronomers, stressed to the ESF "the high technological competence (involving computers, electronics and communications) of young astronomers" and did not see any difficulty in their finding "congenial employment outside astronomy"; whereas Austria, with 31 astronomers, believes astronomers to be "less attractive to industry than other physicists".

Nevertheless—with all these variations—the figures are interesting, if difficult to interpret. There appear to be 2,400 astronomers in the ESF's 16-member Europe, consisting of most EEC countries plus Austria, Greece, Norway, Portugal, Spain, Sweden, Switzerland, and Yugoslavia; there were 1,300 in the US in 1973. 60% of the European astronomers are under 40 years of age, 50% of them 30 to 40. 75% have tenure ("a permanent or semi-permanent post"). The tenured astronomers average 41 years of age; the non-tenured 32. There are some interesting differences between countries. For example Italy has 32 non-tenured astronomers at an average age of 27, but 250 tenured with an average age of 36; while Austria has 18 non-tenured at 32, but only 14 tenured at 50. Italy has increased its numbers of astronomers by 165% since 1965, the report notes.

If the figures are to be believed—and their compiler, Dr M. O. Ottosson, thinks they are accurate to "10 to 20%"—the UK leads Europe in the number of astro-

mers per million inhabitants: 13, to the Netherlands 10. The least astronomical are Portugal (0.6) and Spain (1.3). The UK also has the largest total number—724—with West Germany and France next at 438 and 424. Portugal and Norway have the fewest—5 and 12. Spain has 45 astronomers, for a population of 36 million.

Coming to jobs, the report estimates that there will be some 350 retirements among European astronomers over the next 15 years. "Taking other factors into account, including the creation of new posts", the report estimates there will be some 50 jobs per year. This it compares with the 220 astronomy PhDs emerging each year.

The latter comparison—mild compared with the ratio in some other disciplines such as high energy physics or molecular biology—may have led the Astronomy Committee Chairman, Professor R. Lüst of West Germany, to comment in his preface that "projections for the future show discouraging figures concerning the employment possibilities for young PhDs in astronomy and the development they predict is a rapid decrease of the population in the younger age groups."

But such a view was not without its dissenters. The UK astronomer Professor F. Graham Smith has since remarked that the view is "disastrous" for it may discourage people from taking up astronomy. And in the UK at least Professor Graham Smith considers astronomy to be an excellent training ground, particularly through astronomical technology, for students seeking further employment. Jodrell Bank has been very successful in placing such people, for example. Over the past 20 years, three-quarters of PhD astronomers have found other jobs, Professor Graham Smith believes. The report was held back in its initial version until something of this view could be represented in it; in the event it is represented, in several places, but the preface takes a different line.

Whatever the merits of this case, the astronomy manpower report seems to have raised a question for the ESF. How should it tackle and report on such studies in the future? The ESF has had considerable success with its survey of research expenditure in its member countries, but the accounting procedures in the various countries are a good deal more alike than their systems for assessing manpower. Yet this important issue—in all fields, not just astronomy—deserves European comparison precisely because the experiences in Europe are so different. Only where there are differences can there be lessons.

The ESF is in a perfect position to draw these comparative lessons. But it has a tiny staff (around 10) and cannot be expected to produce the kind of mammoth, comprehensive report that emerges occasionally from the US National Academy of Sciences. Anyway, adopting the philosophy of its President, Sir Brian Flowers, it takes 100 times the effort to produce results that are 10 times as significant. That being the case, and differences being the significant element in this matter, it may have been better if the ESF had produced edited versions of the country reports—despite their differences in approach—with additional notes of comparison and explanation. It would no doubt still be very interesting to ESF members if it were to circulate such a document. □

*A study of manpower in astronomy.*  
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# Science for the people

Scott Schneider, Edward Loechler, Jonathan King and Jonathan Beckwith of *Science for the People* reply to Alvin Weinberg's article 'Science for the people, or by the people?' (3 August, page 410).

ALVIN Weinberg has criticised the position of *Science for the People* on United States cancer policy, and attacked the general idea of public participation in choosing the scientific routes to social goals. He has also commented on the actions of *Science for the People* in the anti-war movement.

Widespread public participation was crucial in stopping US military intervention in Vietnam. We believe that *Science for the People*'s efforts to raise consciousness of the misuses of science and technology — such as chemical defoliants, the electronic battlefield and counterinsurgency research—against the Vietnamese helped to end that war.

Weinberg does not accurately represent our position on cancer research, which has appeared in *Science for the People* magazine (vol VII, September 1975; vol VIII, July 1976). Although the war on cancer channelled billions of dollars into needed biochemical research, the major thrust was limited to tumour biology, tumour virology, and cancer therapy. During the first five years, less than 5% of the research budget was spent on identifying the agents causing cancer. This was in spite of longstanding epidemiological evidence that most human cancer was environmentally caused, and, therefore, preventable (see S. Epstein, *Environmental Determinants of Human Cancer*, *Cancer Res.* **34**, 2425–2435, 1974, and references therein). These environmental agents include such chemical carcinogens as benzidine, vinyl chloride, and other occupational hazards such as asbestos and radiation. For example, New Jersey, the most heavily industrialised state, had the highest cancer incidence in the US for the period 1950 to 1969. The bladder cancer rate in Salem County, NJ, where 25% of the workforce is employed in the chemical industry, is highest in the country. In addition to the epidemiological evidence, there was a well formulated biological model for the role that chemicals may play in cancer, somatic mutation (Ryser, *New Eng. J. Med.* **285**, 721–734, 1971, and references therein); yet the National Cancer Institute (NCI) pursued a research policy concentrating on curing the affected individual to the neglect of decreasing the incidence of the disease.

Although we approve of increased research in the area of nutrition in the cancer programme, we are suspect of Senator Dole's emphasis on the role of diet *per se*. General dietary factors are probably important in the aetiology of cancer, for example the effects of high fat intake. However, a major focus of such research should be on the many carcinogenic food additives which bear little relation to nutritional needs and serve mainly to improve marketability. Other potential dietary carcinogens, including residual pesticides or byproducts of the preparation process, should also be studied. While we support research in these areas, we should not allow a shift in emphasis from cures for cancer to nutritional studies to deflect attention from the other major environmental sources of cancer.

We believe that factors other than the internal state of scientific knowledge determined the systematic underfunding of research focusing on environmental and occupational causes of cancer. For example, in 1952, William Hueper, chief of NCI's Environmental Cancer Section attempted to study the incidence of cancer among chromate workers. The chromate industry management complained that the project was detrimental to their interests and the surgeon general halted the project (L. Agran, *The Cancer Connection*, Houghton-Mifflin, Boston, 1977). Hueper was prevented from doing any occupational studies and was

later dismissed for his persistent efforts in that direction. In 1973, Nixon's Science Advisory Committee issued a major report, *Chemicals and Health*, which supported a continuation of the *status quo* in cancer research priorities. Considering the composition of the committee, which included six representatives of large corporations (Bell, Dupont, IBM, Texas Instruments, Digital Equipment and Turner Construction) and none from labour, environmental, or public interest groups, this conclusion is not surprising. Furthermore, the continued refusal of corporations to allow researchers access to workers' health records has severely inhibited epidemiological studies. At present, the American Industrial Health Council (representing corporate interests) is waging a campaign against the efforts of the Occupational Safety and Health Administration (OSHA) to pass generic standards protecting US workers from occupational exposure to known carcinogens (*Chemical and Engineering News*, 30 January, 1978, pp30-35).

A focus on environmental and occupational carcinogens leads to social policies requiring clean-up of the workplace and elimination of pollution. This generally entails increased production costs and, as a result, reduced profits. In contrast, research into cures for cancer or the viral aetiology of cancer does not threaten corporate interests. It seems likely that this is why corporations and their representatives in government have focused research efforts away from environmental causes of cancer, including chemicals.

In the last few years NCI policy has begun to change. A major factor leading to this change was the struggle to have OSHA pass standards limiting occupational exposure to asbestos, vinyl chloride and other carcinogens. The initially management-oriented OSHA administration was forced to set standards through pressure from trade unions such as the Oil, Chemical and Atomic Workers, public interest groups, notably the Health Research Group, and progressive journalists such as Paul Brodeur. Through these efforts, information about carcinogens became publicly available, and the important scientific contributions of researchers such as Ames, the Millers, Selikoff and Saffioti came to the fore. This public pressure led OSHA, under new leadership, to take up the fight within government to protect its constituency, workers, from cancer. Subsequently, continued efforts by trade unions, environmental and other public interest groups, often acting through their elected representatives (see Leonard Woodcock, United Auto Workers, testimony before the Subcommittee on Health and Scientific Research of the US Congress, April, 1977), forced a change in cancer research policy. Without their contribution, research on the mechanism of carcinogenesis, still in its infancy, would not have developed to the extent that it has.

*Science for the People* believes that the development of scientific knowledge should benefit all sectors of society and that this requires informed public participation. However, in our society, where there are major inequities in the distribution of resources, the forces which generally predominate in the setting of scientific priorities are those with the greatest wealth and influence. What the history of cancer research policy in the US demonstrates is that the means by which scientists set out to achieve a society's goal can be just as heavily politically influenced as the goals themselves. In this case, struggles between corporate interests, on the one hand, and workers and their supporters, on the other, caused shifts in priorities. In fact, the cancer issue also shows that it is only through the pressure from an informed public that certain types of research which were closed off from 'freedom of inquiry' became open. To suggest then, as Weinberg does, that the public has no role in determining the lines of approach which scientists take is to ignore the forces which already shape scientific research.



## Resistance to nuclear power continues. We report on concern over nuclear waste in the United States and Holland and a row over the supply of enriched uranium to Brazil

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*Building Brazil's first nuclear power station (left) and New York (right) which has banned the transport of spent fuel*

# Nuclear waste ban threatens research at Brookhaven laboratory

**David Dickson** discusses how a dispute on the transportation of nuclear wastes reflects scientific and political uncertainties about waste management policy

THE Brookhaven National Laboratory at Upton, Long Island, last week asked New York City's health department for special permission to transport a limited number of spent fuel elements from its High Flux Beam Reactor through the streets of the city.

The request is the latest move in a dispute between the laboratory and the city which has been simmering since 1976, when the city placed a ban on the transport of waste material which could, if unresolved, eventually impinge directly on research activities at the laboratory.

Since 1976, spent fuel from the reactor, which had previously been shipped for storage and processing at the Savannah River Plant in South Carolina, has been stored on site. Plans to ferry the fuel across to the Connecticut shore were frustrated when New London imposed an ordinance similar to the New York ban, and as the storage facilities at Brookhaven begin to fill up, plans to double the size of these facilities are now having to be drawn up.

In the short-term, scientists at the laboratory, which is run by a consortium of nine universities under contract to the Department of Energy, are concerned that the extra money for building new — and, some claim, unnecessary — storage facilities will have to be taken directly out of the laboratory's research budget.

In the longer-term, some are even claiming that if the New York ban

remains in force and problems are experienced in getting agreement on further on-site storage, closing down the reactor, which is a major research facility for fields ranging from materials science to molecule biology, cannot be ruled out. "It seems rather a wild fear, but given the general irrationality with which many of these matters now seem to be dealt, it perhaps becomes more and more real," Dr Martin Blume, associate head of the physics department, said last week.

Brookhaven's problems reflect the extent to which the whole issue of nuclear waste management has become the Achilles heel of nuclear power. The problem is not just the technical one of providing a means of disposing waste fuel, but also involves public acceptability of the means proposed.

Earlier this year, for example, the California state legislature vetoed plans for the \$3 billion Sundersert nuclear power plant after the San Diego Gas and Electric Co had failed to convince a state legislative committee that adequate waste disposal facilities were available. Several other states have since followed California's lead.

In the past, the tendency has been to blame the indecision of policy-makers; one is often told that the problem is "10% science and 90% politics". Responding to such criticism, the Carter administration has recently made a concerted effort to confront the issue, including setting up an Interagency Task Force earlier this year to draw up proposals for an inte-

grated waste management policy.

As the issues have been studied increasingly closely, however, doubts have begun to arise about the priority that has been given to two most conventionally accepted methods of waste disposal — containing the waste in glass and depositing it in salt formations.

Earlier in the summer, for example, the US Geological Survey issued a report by a team of scientists which, although repeating assurances that acceptable underground repositories for radioactive wastes can be constructed, pointed out that there were a number of geologic stumbling blocks which had to be dealt with first.

In the case of buried beds of rock salt, for example, the report says that many questions concerning the behaviour of rock salt and its high solubility must be resolved, such as the fact that relatively small amounts of brine appear to cause a substantial decrease in mechanical strength that would permit movement of waste during a relatively short time.

As far as the primary containment of waste is concerned, a report recently issued by a panel of the National Academy of Science's Committee on Radioactive Waste Management comes to the conclusion that, although glass may be adequate for the implementation of large-scale solidification programmes, "it cannot be recommended as the best choice, especially for older Department of Energy wastes."

The panel, which was chaired by Professor Rustum Roy of Penn State University, says that the preference for glass as a waste form has been mistakenly based on the assumption that low leachability is the major criterion



for solid waste management, and "on a misreading of the 'stability' of glass under repository conditions."

It recommends that, as an alternative to glass, cement-based composites and low-temperature ceramics should be researched vigorously as the prime candidates for solidification of DoE wastes, an approach given wide publicity by Professor A. E. Ringwood of the Australian National University last month, and which has been closely studied in the US for a number of years.

Such challenges to the conventional scientific wisdom on nuclear waste disposal, many of which have resulted from the growing involvement of geochemists into a field previously dominated by physicists and engineers, has led the Department of Energy to broaden considerably the scope of its research options. In addition to salt formations, for example, there are now active programmes investigating the suitability of other geological media such as basalt, granite and shale.

At the same time, the department is adopting a more holistic approach to the waste management problem. Dr John Deutch, head of the Department's Office of Energy Research, told a Senate committee two weeks ago that the selection of a particular site for its environmental qualities could be more important than the choice of a particular medium.

"It is very important that the entire environment of a site is evaluated, in particular its chemical, geological and hydrographical characteristics", Dr Deutch told the committee, adding that "the problems of finding a site, even for just assessing, are formidable."

Such problems are illustrated by the Department's current plans to construct a Waste Isolation Pilot Plant (WIPP) in salt beds near Carlsbad in New Mexico. The nuclear industry is keen that the department should move ahead rapidly with its plans for WIPP, thus demonstrating that the safe disposal of radioactive waste is feasible.

However, local environmental groups in New Mexico, drawing attention to the USGS and other reports which point to current gaps in knowledge about the behaviour of salt — as well as the problems of later retrievability of the waste from salt deposits—are challenging the department's plans on the grounds that other geological media might be more appropriate for such a demonstration plant.

Both the frustration of the nuclear industry, and the concern of environmentalists, stems partly from the fact that the more research that is done into methods of waste disposal, the more scientists are realising that important gaps in knowledge still remain.

A recent report prepared by the



### The size of the waste problem: America's nuclear reactors

Map taken from *Oceanus*, adapted from data supplied by Nuclear News, September 1976 and from US ERDA

Office of Science and Technology Policy for the President's Interagency Task Force, for example lists "numerous limitations of our knowledge" in hydrogeology, geochemistry and rock mechanics, "relatively young sciences with little experience in making or evaluating predictions that cover time frames even as short as a few decades".

The OSTP report adds that, despite such limitations, "we believe that such gaps in our knowledge need not rule out successful underground containment of radionuclides for periods of many thousands of years". Yet while such gaps exist, the question of the "adequacy" of proposed disposal—and even transportation—methods remains open to active debate.

It was the lack of federal regulation concerning the transportation of nuclear wastes, for example, that led the US Department of Transportation to issue a decision earlier this year supporting New York City's right to impose the ban that has prevented Brookhaven from shipping its spent fuel to South Carolina, and agreeing that, under the terms of the Hazardous Material Transportation Act, the ban could not be pre-empted by federal action.

The laboratory is at present contesting the constitutionality of the ban. "The laboratory does not acknowledge the validity of New York City's ban and New London's ordinance on the transportation of spent fuel elements through these cities", according to Mr Michael Goldman, an attorney for the universities which operate the laboratory. "But we would like to begin shipping spent fuel elements this fall to ensure that there is no interruption of research at the reactor."

Opponents of the ban point to a recent report from the Sandia Laboratories claiming that the transport of radioactive waste through urban areas poses a relatively low risk. Supporters,

such as Friends of the Earth, claim that this study was coloured by the fact that it was commissioned by a government agency keen to validate its claims that federal legislation should pre-empt local regulations.

Some guidance will no doubt be provided by the Interagency Task Force when it presents the results of its deliberations to President Carter at the beginning of October; the Department of Transportation, for example, is preparing a study for the task force on the transportation of waste, and the Brookhaven case should figure prominently in its deliberations.

Ironically, current work at the reactor involves a variety of research projects in the health and environmental fields, including not only a study of trace elements and pollutants in the environment, but also an analysis of air samples for the New York State Department of Health. □

### Nuclear moratorium

WISCONSIN has become the fourth US state to place a moratorium on the future construction of nuclear power plants in the light of current uncertainty over running costs, the availability of nuclear fuels, and safety aspects of waste disposal and decommissioning.

In announcing the moratorium last Friday, the state's Public Service Commission joined similar bodies in three other states—California, Iowa and Maine—which are pressing the Federal Government for firm guidance on such issues.

Conceding that nuclear power had an "impressive record" in Wisconsin, State Governor Martin Schreiber said its performance in other states "produces doubts that it is economically preferable to other forms of energy, energy conservation and alternative renewable energy sources. □

# Dutch delay decision on nuclear expansion...

HOLLAND is unlikely to build any more nuclear power plants until 1990. Discussion of an expansion programme—and in particular problems of uranium enrichment and waste disposal—is now into its fifth year. According to a recent government decision, the debate is to continue over the next two years and it looks as though a decision on the future of nuclear energy in the country will not be made before 1982.

The limitations of Holland nuclear energy—the country has two plants, both small (477 MW and 56 MW)—were recognised by the previous government in 1974. Three more plants, each of 1,000 MW, were called for, but plans for them were shelved two years later.

The urgency of the energy problem was highlighted by Mr Van Aardenne, Minister for Economic Affairs, in a recent letter to Parliament. Almost all the country's 14,500 MW energy capacity would have to be renewed by the end of the 1980s. By then, natural gas, which at present fuels three quarters of the country's power stations, would have to be reserved for domestic use rather than generation of electricity. But shifting totally to coal-fired power plants would mean consuming 14 million tons of coal a year by 1995 (compared with only a million tons now), rising to 23 million tons a year by the end of the century. Consequently, a quick decision was needed on the possible contribution of nuclear energy, the Minister said.

Disposal of nuclear waste will figure prominently in the two years of public debate now ahead. It was this issue that caused postponement in 1976 of the earlier plans to build three more nuclear power stations. Moreover, a guarantee of acceptable means of waste disposal and storage before building new plants was also part of the election platform of the new government elected last year.

The government is to speed up its research on waste disposal. Five sites for storage salt domes have already been provisionally picked but research is being hampered by the refusal of the local authorities involved to co-operate. Even so, a team of government experts has said the first storage mine could not be ready until 1995. □

## ...but let safeguards slip on Brazil deal

THE Dutch government has agreed to start delivering enriched uranium to Brazil around 1981—without complete international safeguards. Initially, the Dutch had wanted an assurance that international control of plutonium after reprocessing would be in force at the time of the first delivery. Now, however, in a decision in parliament in June, they have agreed to start delivering the uranium if safeguards are likely to be in force by the time reprocessing starts, probably in 1986.

The Brazil saga started in October 1976 when the former government of Social Democrats, Christian Democrats and a few radicals decided to expand Urenco's ultra-centrifuge uranium enrichment plant at Almelo. Its capacity was to be increased from the present 200 tonnes to 1,200 tonnes per year. The expansion is only partly based on the Brazilian order for uranium; but this in turn is coupled with the 10 billion DM transaction for nuclear technology between West Germany and Brazil.

Brazil's order includes eight 1,200 MW nuclear power plants, a reprocessing plant, and enrichment technology based on the German 'nozzle process'. The nozzle process is not yet operating commercially, so Brazil has to begin by importing enriched uranium. German participation in Urenco meant that a delivery from Almelo seemed possible.

As with all international nuclear sales there had to be agreement on safeguards against weapons-buiding, and in February 1976 Germany, Brazil, and the IAEA reached a trilateral agreement.

But the agreement threw the Netherlands into a political storm. Brazil appeared to have given no safeguards

or guarantees for the import of enriched uranium in 1981, but only agreed to accept a plutonium storage regime under IAEA control in about 1986 when the first uranium will be reprocessed. And if Brazil would agree then, why could it not agree now?

The Dutch parliament repeatedly postponed a decision on what Holland should do. The radicals were threatening to break up the government coalition over the issue. A final decision on a plutonium storage regime and IAEA safeguards covering the whole nuclear cycle (so-called 'full-scope' safeguards) was expected in about March 1976, but was still pending at the end of last year. The former Dutch foreign minister, Mr Van der Stoep, declared later that the problem of IAEA inviolability and right of say on the movement and storage of plutonium was still in discussion with the Brazilians when the government changed in December 1977.

The new coalition of Christian Democrats and Conservatives presented Parliament with an agreement between the Urenco partners and Brazil, similar to the February 1976 agreement. But at the end of January 1978 parliament adopted a motion for a more definite agreement. It approved the expansion of the Almelo plant and also decided that no uranium from Almelo could be exported to non-NPT countries unless strict safeguards were agreed in advance. A motion, carried by an overwhelming majority in the Lower House, stated that an export licence for uranium could be granted only if a plutonium storage regime under the control of the International Atomic Energy Agency (IAEA) was in

force or an *ad hoc* agreement on the storage of plutonium was reached between Holland and Brazil. The government agreed that it would respect and carry out Parliament's decision.

The affair received a new impulse. Discussions and negotiations with Germany and Britain started up again; but these failed.

At the European summit conference in Copenhagen in the Spring, Chancellor Schmidt had no scheduled meeting with the Dutch prime minister Van Agt. The Dutch foreign minister, Van der Klaauw, was refused a meeting with his Brazilian colleague, who was in Bonn, and the Germans threatened to build their own enrichment plant across the Dutch-German border to deliver the uranium to Brazil when the tripartite treaty ends in 1981. The Dutch could then drop out if they still refused to export the uranium on the basis of the agreement made last January. The Germans were said to prefer to continue without the Dutch—who were said to weigh moral and political considerations more heavily than economic ones.

However, some German politicians realised it would be a political error if Germany were not to extend the Urenco agreement beyond 1981. The United States favoured the Netherlands remaining in Urenco, but thought that the safeguards should be 'full-scope', covering the whole nuclear cycle, rather than just covering the storage of plutonium.

Apart from the political implications there have also been doubts about the commercial future of Urenco. The original plan was to expand the Almelo and Capenhurst plants to a total joint



capacity of 5,000 tonnes by 1985. Now, however, a capacity of only about 2,000 tonnes per year is foreseen for 1984. Although the Brazilian order is only 10% of the uranium now on order, it will be sold at a profitable price, which is more than can be said of the previous German orders. At the end of March 1978 it became known that Dutch banks were excluded from financing the Brazilian uranium order because only the German and British governments are providing the guarantees.

In the first six months of this year, the matter was discussed extensively four times in the Dutch parliament. In March 30,000 people, some from Germany, demonstrated in Almelo. Two weeks later, 50,000 people from 28 eastern-bloc and western countries took part in a demonstration in Amsterdam against the neutron bomb and nuclear energy. And at the end of June The Hague was the scene of a similar demonstration.

In the meantime it came into the open that the IAEA, at least, *had* been in favour of a plutonium storage regime. Although such a regime cannot

be set up for a few years, an *ad hoc* regime in Brazil on the basis of an available blue print seemed feasible. What was needed to put the scheme into practice was the political will of the countries concerned, said IAEA deputy secretary general Fischer on Dutch television. A proposal for such a regime would be judged positively by the IAEA board on which 35 countries are represented. However, so far the Urenco partners have not requested an international storage scheme for plutonium.

In an international safeguards system, there would be regional depots for plutonium but probably not in Brazil. In an *ad hoc* regime with Urenco such a depot could be in Brazil. Brazil will therefore be against a world storage regime.

The whole affair came to an end with the government's decision at the end of June. The motion adopted by the majority of parliamentarians in January required that a storage regime be established before the first delivery of uranium takes place. The final agreement, which states that a storage regime must be available when re-

processing starts, is less stringent in its requirements.

Now the only reservation is that there should be sufficient certainty, at the moment of delivery of the first uranium, that before reprocessing starts an agreement would be reached on a storage regime. However, parliament may find difficulty in judging the likelihood of such an agreement being reached, leaving the government fairly free to manoeuvre.

What has happened is what Germany and Britain wanted, say the opponents, who also tried other moves to avoid this agreement. The social democrats presented a bill to regulate the future export of all nuclear material, as in the United States; if accepted the bill would also include the deliveries to Brazil.

The net result of the negotiations is that the opposition feel that the whole affair has been a blow to democracy and to attempts to create a proliferation proof society. "We move from co-responsibility to complicity", the spokesman of the opposition (social democrats) said in parliament.

**Casper Schuurig**

## US plans to ban the use of nitrites in food

THE US government is planning to introduce a gradual ban on the use of nitrites in food, following the results of studies carried out at the Massachusetts Institute of Technology indicating a "strong suggestion" that nitrites are carcinogenic.

Steps have already been taken to reduce the use of nitrites as preservatives, particularly in cured meat such as bacon and ham, because of evidence that, when cooked and eaten, they can combine with other chemicals to cause carcinogenic nitrosamines.

The latest study, however, which was conducted by Dr Paul Newberne of MIT, is the first to link nitrites directly with cancer. In the study, 13% of rats fed sodium nitrites developed tumours of the lymph system, compared to only 8% of those not fed nitrite.

The US Department of Agriculture and the Food and Drug Administration have called the results "statistically significant", and said in a statement last week that the study "leads us to the concern that nitrites may increase the incidence of human cancer."

Under current legislation, both agencies have a range of powers to take action against any food additive suspected of being carcinogenic. However in this instance the government is refraining from acting precipitously partly because of the embarrassment caused by its attempts to ban the use

of saccharin, a move on which Congress has since imposed an 18-month moratorium.

An additional factor is that the use of nitrites considerably cuts down the danger of botulism poisoning from preserved meats, and permits greater freedom in shipping and storage. "We must weigh the risk associated with nitrites added to food against the health risk of not adding it" the two agencies said in their statement.

Because of the public health angle, the federal government is considering ways of introducing a gradual ban on the use of nitrites in circumstances where protection against botulism toxics is important, and where alternative safety techniques need to be developed and implemented. There is likely to be a quicker ban on products

that are always cooked thoroughly before they are eaten, since this generally eliminates the chances of botulism poisoning.

In addition to banning nitrites, the government is also proposing to ban the addition of nitrates to processed foods, a move which could, for example, have an impact on the import of cheeses.

The economic impact of a ban on nitrites could be even bigger than that of the proposed ban on saccharin. According to the American Meat Institute, a nitrite ban could "all but destroy the US hog industry, since nearly 70% of our pork ends up in processed meat products—mostly cured meats." Annual consumption of such products in the US is currently about \$12.5 billion.

In the light of such a potential impact, measures to forestall a ban on nitrites were introduced into the US Congress last Thursday by Representative James Martin of North Carolina and Representative William Wampler of Virginia.

The two Congressmen are seeking to block any ban until at least three months after the government completes its current investigations into other suspected carcinogens, including saccharin. The issue is not likely to be debated this year, but could become a hot topic when the new Congress meets after the fall elections.

**David Dickson**



"If I don't get him with cancer, I'll get him with botulism!"



# Hungary starts to tackle pollution

HUNGARY has recently set up a new National Council for the Environment and Nature Protection. The council has the status of a State Committee under the Council of Ministers, and a membership which includes those ministers and heads of national bodies concerned with environmental protection, and scientists and specialists.

Environmental protection has been a long-standing matter of national concern in Hungary—and the history of governmental counter-measures is almost equally long. On formal and prestige occasions, it is even possible for orators and publicists to hark back to the days of the Dual Monarchy and cite the Public Health Act of 1876, which *inter alia* empowers the authorities to “use compulsions to remove everything which pollutes the air, the soil or the waters . . . and to establish anything which promotes public health”.

Unfortunately, of recent years, concern has not always been matched by action. The new law on the protection of the human environment, which was presented to the National Assembly in 1974, was not in fact passed until March 1976. (Press releases at the time of passage of the Bill hinted that the delay was due to incorporating the recommendations of the Helsinki Final Act into the final format.)

Similarly, although a National Anti-pollution Committee was set up in 1969, the definition of its terms of reference proved a very slow process, and almost a year later the newspaper *Nepszava* pointed out that no ruling had yet been given as to the committee's authority on the national level. Not surprisingly, law-suits on questions of pollution were not uncommon: sometimes, indeed these verge on the Gilbertian, as in the 1972 action against the Borsod Cement and Lime Works. The dust emitted by the Hejocsaba unit of the works was emitting pollutant dust which was alleged to affect the quality of fodder, the composition of the soil, damage the fish hatcheries and even affect pollination. The case turned on the precise legal definition of “activity fraught with excessive danger”. (An equally ludicrous situation arose in 1977, when, although the Debrecen Synthetics Plant was emitting 4–5 times the maximum permitted level of lead, the plant went on operating, since no one seemed sure who should be responsible for closing it down!)

Equally ironic is the case of the Lake Balaton Area. The Little Balaton, once a part of the main lake, is now connected to it only by the river Zala

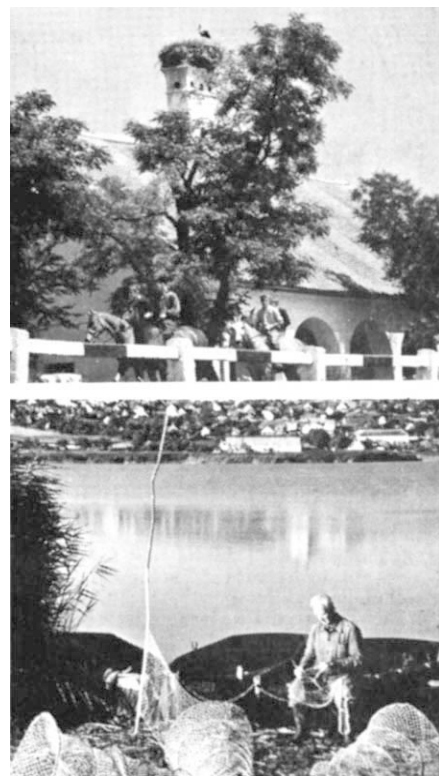
and is largely reed-grown, providing an excellent habitat for such rare species as the little egret (*Egretta garzetta*), the bittern (*Botaurus stellaris*), spoon-bill (*Platalea*), and some of the last tufted herons (*Egretta alba*) in Europe. Yet Lake Balaton proper, until the early 1970s, was itself a ‘reservation’—for foreign motor boats, which by then had been banned from almost all the lakes of Europe. Apart from the pollution hazard to the lake itself, the noise and atmospheric pollution from the boats caused considerable outcry in the media, which urged the banning of the offending vessels.

During the 1970s a number of practical measures were introduced. A system of progressively increasing fines were imposed on industrial enterprises which contravened air pollution regulations—the proceeds going to anti-pollution R and D and to the installation of anti-pollution equipment.

The maximum sulphur content of domestic solid fuels in central Budapest was restricted to a maximum of 1%—thus creating one of the few ‘smokeless zones’ in Eastern Europe. The Patriotic People's Front—an umbrella organisation for political mobilisation—was drawn into the programme. One of its first suggestions—at the Visegrad Conference of 1973, was that courses in environmental protection should be included in curricula at all levels of education.

By the time the Environmental Protection Act was finally passed in 1976, Dr Karoly Garamvolgyi, Deputy Minister of Education, could inform the press that “instruction is given in environment protection and anti-pollution measures on every level of education from the kindergarten and first-level education through secondary schools and skilled workers’ training schools, to the third-level in our colleges and universities, and finally in postgraduate training.” Nevertheless, he thought that more could be done for the 3 to 6 age group. The teachers’ training colleges, he said, were still not putting sufficient emphasis on these problems; however, a new primary school syllabus had recently been worked out, “in which the subject is treated in integration with other subjects”.

In spite of all efforts, the situation, as reported by the Hungarian radio last June, is still far from satisfactory. The annual damage from pollution equals 2.5 to 3.0% of the ‘national income’ (a Marxian term roughly equivalent to the net material product, but with certain ‘non-productive’ factors



All clear at Hortobagy and Lake Balaton but industrial pollution is the main problem

omitted). Some 14,500 ha of land are lost to agriculture each year, largely due to the spread of industry and mining. Some 50 to 150 tonnes of solid industrial waste (fly ash, heavy metal dust, slag, etc) pollute every sq km of soil per year.

Logistic factors and lack of resources undoubtedly play a part here. Thus in 1971, when there were a number of problems as to the best means of purifying industrial wastes, an experimental programme was introduced at Kecske-met, using unpurified industrial wastes to irrigate crops.

Nor is Hungary's record entirely gloomy. In 1973, the Swiss-based Goethe Foundation awarded the Europa prize to Laszlo Madas, Director of the Pilis Forest, for his ‘outstanding services’ to ecology.

This award, perhaps, is significant; to date Hungary's major successes have been in the establishment of nature reserves and protection areas, rather than in the combatting of industrial pollution. The establishment of a new breeding centre for bustards, the setting up of the National Park of Hortobagy, with its aboriginal grey cattle, or the protection of the creeping sand-hills of Kiskunsag—all this appears to come far more easily to the Hungarian planners than the combatting of industrial pollution. It is to be hoped that the new Council, with its co-ordinating powers, will be able to deal with this far more insidious problem.

Vera Rich

# correspondence

## Racism refuted

SIR,—Many readers of *Nature* will have seen some of the extensive press coverage given to the campaign launched in the UK by the, avowedly racist National Front earlier this year to recruit members from schoolchildren and the young unemployed. This has included, according to the NF leadership, the distribution of up to 350,000 leaflets headed 'How to Spot a Red Teacher'. A central theme of this leaflet, and of a large supporting pamphlet on 'How to Combat Red Teachers' is the allegation that there are scientifically proven biological and psychological differences between races. As the leaflet puts it, under the heading 'The Racial Equality Lie': "Tell the Red Teacher that top scientists like Jensen and Eysenck say this is rubbish. Scientists say that races are born different in all sorts of ways, especially in intelligence. This is because we inherit our abilities genetically".

The centrality of scientific racism to the NF's propaganda is indicated, too, by the comment of their national organiser, Martin Webster (who has publicly boasted of creating a "well-oiled Nazi machine" in Britain) that "the most important factor in the build-up of self-confidence amongst 'racists', and the collapse of morale among multi-racialists was the publication in 1969 by Professor Arthur Jensen in the *Harvard Educational Review*, (cited by Martin Walker in *The National Front*, Fontana, 1977, page 169)".

Professor Eysenck has given personal interviews to *The Beacon*, the newspaper of the now defunct, NF-related organisation, the National Party.

Many biologists and psychologists, myself included, have taken issue with Professor Eysenck and Jensen over their views on the genetic basis of intelligence and its class and race distribution. However, the claims made by the NF propaganda, in the names of Eysenck and Jensen, go far beyond anything that even they themselves have claimed. The openly racist intent of the NF, with its lineage stretching back to the 'Aryan biology' of the 1930s, must be repugnant to the majority of scientists and lay people alike. It would be a service to the cause of race relations in Britain, therefore, if Professors Eysenck and Jensen were to publicly and unequivocally dissociate themselves from the NF and its use of their names in its propaganda. Can I, through the courtesy of your columns, therefore, ask them to do so?

STEVEN ROSE  
*The Open University, UK.*

SIR,—Professor Rose suggest that I have given personal interviews to *The Beacon*; this is untrue, although I am sure he made the allegation in good faith.

In a letter to *The Times* published earlier this year, I made it quite clear that I deplore the linking of my name and that of Professor Jensen, with the claims of the National Front for white supremacy and 'racism' generally. As I made clear in my original book on

*Race, Intelligence and Education*, and on numerous other occasions, I am absolutely opposed to any form of racism, and believe that the scientific evidence unequivocally points to the need to treat each person individually in terms of his own personality, abilities, and achievements, not as a member of a racial group, or a particular sex, or class.

A denial of this proposition being the essence of racism, I believe that the large overlap in measures of ability always found between different races destroys, rather than supports, the basis of racism. It is source of considerable distress to me that the National Front has attempted to use my name and Professor Jensen's in their propaganda, and I am happy to take up Professor Rose's invitation to dissociate myself from the National Front and any other explicitly racist organisations. No-one familiar with Professor Jensen's or my own writings could possibly misinterpret our arguments about the mean differences between various racial and other groups with respect to intelligence as implying the kind of policies advocated by the National Front; I made this clear in my original publication, and have done so since.

H. J. EYSENCK  
*University of London, UK*

SIR,—Nothing has hindered progress toward a scientific understanding of the nature and causes of human differences in general mental ability individual differences and statistical differences between races than the gross and deliberate distortions of the available evidence by the extreme left and the extreme right. Both are equally guilty and both are equally offensive to all who seek a scientific understanding of human variation. Unfortunately, serious students of the subject are often forced to duck from the ideological cross fire.

Extremists of the left and right are alike in regard to the heredity-environment controversy. They are both anxious to promote and to gain public acceptance of a particular dogmatic belief about the nature of racial differences, even when the scientific evidence is inadequate or contradictory. They both officially abhor a publicly agnostic stance regarding the scientifically still open questions. They both distrust the public with access to relevant knowledge and discussion concerning social class and racial differences. Most fundamentally, they both disdain human individuality. The leftists ideologically prefer to deny real differences in ability between individuals if the individuals are of different racial background; the rightists ideologically ignore differences between individuals if they are of the same racial background and treat human differences exclusively as differences between groups, obscuring the wide range of differences within each group and the great overlap of trait distributions among all human groups. Much overlap is found not just for mental ability, but for all important behavioural traits.

Recognition of individual differences

and respect for individuality are the keystone of a free society, which both extremes of the political spectrum would destroy if they had their way. These ideologues have no real interest in scientific aspects of the 'nature-nurture', 'race difference', or 'IQ' controversies.

I have written a summary of my overall assessment of the issues in this complex field in "The Current Status of the IQ Controversy", an article recently published in the *Australian Psychologist*. In it, I stated:

"Research findings can have implications for social policies and practical applications only in relation to goals and values of the society. These implications do not flow directly from the scientific facts themselves . . . [T]he well established findings of a wide range of individual differences in IQ within all major racial populations and the great amount of overlap of their frequency distributions contradicts the racist philosophy that individuals of different races should be treated differently, one and all, only by reason of their racial differences. Those who would accord any treatment to individuals solely by virtue of their race will find no rational support from any of the scientific findings or theories of modern differential psychology. Man's genetic nature insures individuality, and any doctrine that is built on a denial of this fact is simply at odds with reality . . . My concept of justice requires that the fact of statistical differences between racial populations should not be permitted to influence the treatment accorded to individuals of any race—in education, employment, legal justice, and political and civil rights.

. . . Righting the past wrongs of racial discrimination can be accomplished best . . . by prohibiting racial discrimination in any form, by legal sanctions when necessary, and by seeking equal educational opportunities for members of minority groups who have been denied them in the past, so they can compete fairly for employment, technical training, or higher education, without conceding dispensations.

" . . . Since we are still far from a scientific consensus as to the causes of [racial] differences in educability, the only intellectually warranted official position of educators and governmental policy makers must be one of *open agnosticism* as to the causes, rather than the doctrinaire naive environmentalism that has so long prevailed as official policy. If scientific agnosticism is deemed unsatisfactory as a permanent state of affairs, and scientists are drawn to the challenge of reducing the heredity-environment uncertainty, they have no choice but to continue the pursuit of normal science in the IQ controversy. In the history of intellectual conquest, agnosticism concerning socially important natural phenomena has always been a highly unstable condition; it invariably gives way either to dogmatic belief or to scientific knowledge."

ARTHUR R. JENSEN  
*University of California,  
Berkeley, USA*



# news and views

## New data from solar oscillations

from Douglas Gough

New analyses of the observations of solar oscillations have posed more problems about the internal structure of the Sun. At a meeting on Highlights of Solar Physics held recently in Toulouse, Henry Hill (SCLERA, Arizona) presented evidence that of the oscillations he had reported previously at least those with periods of 45 min and 1 h contain components that are internal gravity waves. He compared signals obtained from integrating light from different extents of the solar limb and was able to deduce that there is significant power in modes with horizontal wavelengths between  $1/40$  and  $1/20$  the radius of the Sun. If these modes were standing acoustic waves their frequencies would be at least twice those observed, whereas gravity modes, or  $g$  modes, could have the observed frequencies.

The problem this observation raises is that if the structure of the Sun were as most solar physicists believe, with a convective envelope penetrating some 20% of the solar radius beneath the photosphere, one would not expect to see the modes Hill reports. Wojtek Dziembowski emphasised at the Toulouse meeting that typical gravity waves with so short a horizontal wavelength are severely confined within the radiative interior of the Sun, being attenuated through so deep a convection zone by a factor of a million or more. Therefore they could not possibly be seen at the photosphere. How can this difficulty be resolved?

One potential explanation, as Jurgen Christensen-Dalsgaard has pointed out, is simply that the modes are not so confined beneath the convection zone. There is a class of solar oscillations; formally classified as  $g$  modes, whose amplitude is greatest at the surface. They are those overtones which, irrespective of the detailed structure of the Sun, have frequencies close to  $\sqrt{gk}$ , where  $g$  is the acceleration due to gravity and  $k$  is the horizontal wavenumber of the mode. The modes have

the same frequency as surface waves on deep water, and are dynamically similar. Unfortunately such modes with wavenumbers corresponding to those reported by Hill would have periods between about 25 min and 35 min, which are too short to account for the observations.

A second possibility is that the solar structure is actually rather different from the so-called standard models. Some years ago Paul Joss (*Astrophys. J.* **191**, 771; 1974) argued that the Sun was formed with a low heavy element abundance and subsequently suffered contamination of its surface by infalling interstellar material. If that were the case the Sun would have a very shallow convection zone. It would support a class of  $g$  modes trapped just beneath this zone which have substantial amplitudes in the photosphere, and have periods and wavelengths compatible with Hill's observations.

At first sight this idea sounds attractive, especially since it also leads to a low neutron flux consistent with Davis's measurements. But there are difficulties in explaining how the Sun could have formed with so low a heavy element abundance that would be required to explain the present luminosity. Potentially more serious an objection, however, comes from Franz Deubner's measurements of the diagnostic diagram for the 5-min oscillations, recently confirmed by Rhodes Jr, Simon and Ulrich (*Astrophys. J.* **218**, 521, 901; 1977) which seems to be reproducible theoretically only with solar models having convection zones even deeper than those of the 'standard' models. The theory of the 5-min oscillations, however, is not yet sufficiently developed for us to know how serious an obstacle this raises for advocates of the shallow convection zone.

A result we have been waiting for since Deubner's first publication of the diagnostic diagram is the rotational splitting of the 5-min modes, which

measures the mean Coriolis force experienced by the oscillating fluid. This has now been provided by Deubner, Rhodes Jr. and Ulrich and will be published soon in *Astronomy and Astrophysics*. Unfortunately there is much scatter in the data, but it has at least been possible to deduce that the solar angular velocity at the equator increases with depth. This result is consistent with our previous deductions from comparisons of the photospheric rotation deduced from Doppler shifts and the more rapid rotation of magnetic features, which presumably reflects the motion near or below the base of the supergranules.

Rotational splitting may also have been detected in the 2 h 40 min oscillations reported by Severny, Kotor and Tsap (*Nature* **259**, 87; 1976). This is of particular interest because the long period oscillations penetrate deep into the solar interior, unlike the 5-min modes which are confined to within about 10,000 km of the photosphere.

At the Toulouse meeting the Crimean astronomers reported a modulation in the amplitude of the 80 min Fourier component of these oscillations with a period of about 27 d, or possibly half that value. If this results from rotational splitting of nonaxisymmetrical  $g$  modes it could have important implications concerning the mean rotation of the entire Sun, although whether it implies slow or rapid rotation depends on the degree of the surface harmonic characterising the mode. This has not yet been measured. The result would have a bearing on the solar oblateness, and in this context it is interesting to note that the rotationally induced precession of the  $g$  modes deduced from the Russian observations could be comparable with the precession of the apparent oblateness of the Sun reported by Dicke (*Solar Phys.* **37**, 271; 1976).

These new results have not changed our ideas about the solar structure, but they have added to the data that must constrain our theoretical models. □

Douglas Gough is at present at the Observatoire de Nice.

\* The proceedings of the meeting in Toulouse on Highlights of Solar Physics have been edited by J. Rösch and will be published by the CNRS in October.

# Epstein-Barr virus as the cause of a human cancer

from M. A. Epstein

BURKITT'S first account of the malignant lymphoma which now bears his name (Burkitt *Brit. J. Surg.* **46**, 218; 1958) attracted little attention. However, Burkitt's lymphoma soon acquired outstanding significance when Burkitt showed that its distribution in Africa is determined by climate; for, the fact that the incidence was influenced by temperature and rainfall suggested that a biological agent such as an oncogenic virus was concerned in causation (Burkitt *Brit. med. J.*, **ii**, 1019; 1926; *Nature* **194**, 232; 1962), although the original hypothesis as to how this might be has required revision.

The concept of a viral aetiology for endemic Burkitt's lymphoma has gained steadily in strength with the discovery of Epstein-Barr virus (Epstein *et al. Lancet* **i**, 702; 1964) and the gradual demonstration of its close association with the tumour (Epstein *Prog. exp. Tumor Res.* **21**, 72; 1978), paralleling the relationship of known oncogenic animal DNA viruses to the tumours they induce. Thus, Burkitt's lymphoma patients have high titre antiviral antibodies with a specific pattern varying with disease events, and every tumour cell carries the virus DNA with at least one molecule linearly integrated into the host cell genome giving expression of virus-coded neoantigens; in addition, the virus transforms normal human cells *in vitro* into continuously growing lines and induces malignant tumours experimentally in subhuman primates (Epstein & Achong *A. Rev. Microbiol.* **31**, 421; 1977). Although Epstein-Barr virus therefore seems to be the cause of endemic Burkitt's lymphoma, ethical constraints make direct proof of this hard to obtain in humans. In any case, the virus cannot act alone in the aetiology of the tumour, since the virus occurs throughout the world (Henle & Henle *Prog. exp. Tumor Res.* **21**, 19; 1978) whereas a high tumour incidence is restricted to parts of Africa and New Guinea with specific weather patterns. A climate-dependent cofactor seems essential in determining the distribution of the tumour, and observations of many kinds indicate that this cofactor is hyperendemic malaria (Burkitt *J. natn. Cancer Inst.* **42**, 19; 1969; *A. Rep. Internat. Agency Res. Cancer, Lyon*, 78, 1977).

Strong new support for a causative role for Epstein-Barr virus in endemic

Burkitt's lymphoma has now been provided by an important prospective study in a high incidence area for the tumour in which WHO's International Agency for Research on Cancer has been following 42,000 children serologically since 1972 in the West Nile District of Uganda (de Thé *et al.*, this issue of *Nature*, page 756). The scale of the undertaking and the efficiency of the modest logistics employed are remarkable, while the absence of interruption in this particular region provides an unusual criterion of WHO prestige.

This prospective study has demonstrated that children destined to develop endemic Burkitt's lymphoma have long been infected by the virus and show an important difference in their response to it, namely unusually high titres of antibodies to the virus capsid antigen compared with controls. The data on these high antibody titres have permitted the calculation of the risk factor attached to such a response to the infection—the risk of developing endemic Burkitt's lymphoma was approximately 30 times higher for children with a virus capsid antigen antibody titre two doubling dilutions or more above those of the control population.

Such a risk factor is very striking and is indeed appreciably greater than that accepted as establishing an aetiological relationship between heavy smoking and carcinoma of the bronchus (Doll & Peto *Brit. med. J.*, **2**, 1525; 1976; *Roy. Coll. Physicians London, 3rd Rep. Smoking or Health*, Pitman Medical, London, 54, 1977).

The ways in which the virus may interact with the essential climate-dependent cofactor of hyperendemic malaria to bring about malignant transformation have become clearer through other kinds of investigation. It is well known that only B lymphocytes have receptors for the virus (Pattengale *et al.*, *Lancet* **ii**, 93; 1973; Jondal & Klein *J. exp. Med.* **138**, 1365; 1973), and also that malaria is both immunosuppressive and a stimulator of B cell proliferation (Salaman *et al. J. gen. Microbiol.* **59**, 383, 1969; O'Connor *Am. J. Med.* **48**, 279; 1970; Greenwood *et al. Lancet*, **i**, 169; 1972); persistent malaria might therefore stimulate and maintain an unusual supply of lymphoid cells especially liable to undergo malignant change when infected by the virus. Such cells could be a specific type of B cell or B cells at a particular early stage of differentiation. There is evidence now that certain subclasses of B lymphocytes constitute a special target for transformation by Epstein-Barr virus *in vitro* (Katsuki *et al. Virology* **83**, 287; 1977; Steel *et al. Nature* **270**, 729; 1977), whilst the lack of differentiation shown by the cells from acute

leukaemias (Greaves *et al. in Immunodiagnosis of leukaemias and lymphomas* (ed. Theirfelder, Rodt, Thiel) Lehmanns Verlag, Munich, 1977; Janossy *et al. Leukaemia Res.* **1**, 289; 1977) suggests that cells malignantly transformed to give endemic Burkitt's lymphoma might likewise be at an early stage in differentiation.

Where Burkitt's lymphoma is common Epstein-Barr virus affects everyone at an early age and hyperendemic malaria affects over 50% of children, yet of these doubly-infected individuals only a rather small number develop tumours, perhaps because of genetic predisposition, or very early infection, or a particular sequence in the timing of infection in relation to the acquisition of malaria.

Direct proof that Epstein-Barr virus causes Burkitt's lymphoma can only be obtained by showing that vaccination against the virus decreases tumour incidence. The new data lend added support to this proposal and to attempts to control by vaccination the numerically more important Epstein-Barr virus associated human tumour, nasopharyngeal carcinoma. □

## International study of short-term carcinogenicity tests

AN international study, now in progress, is looking at the reliability of various *in vitro* assay systems such as the Ames test in predicting carcinogenicity.

In this study, which is supported by the UK Medical Research Council, UK Health and Safety Executive, ICI, the US National Institute of Environmental Health Sciences and the US Environmental Protection Agency, the response of about 25 assays (see box) will be determined for 42 carcinogens and non-carcinogens. Investigators will be unaware of the identity of the chemical they are testing. All available carcinogenicity data on the selected compounds will be reviewed by an independent panel, as the definition of non-carcinogenicity is central to the study. It is recognised that the results of this study might possibly cause re-evaluation of the carcinogenicity of some chemicals.

The Salmonella reverse mutation assay (the Ames assay) has gained prominence as a test of carcinogenic potential and will be concentrated on both in this study and in a related study organised by the US National Cancer Institute—in which a smaller number of assays are being evaluated with a larger range of chemicals. Be-



**Prokaryotic systems**

## Repair deficiency assays

*Bacillus subtilis*—rec<sup>-</sup>  
*Escherichia coli*—rec<sup>-</sup>  
*Escherichia coli*—pol A<sup>-</sup>

## Point mutation assays

*Salmonella typhimurium*/microsome  
 (Ames test)  
*Salmonella typhimurium*  
 8-azaguanine resistance  
*Escherichia coli* WP-2  
*Escherichia coli* 343/113

**Eukaryotic systems**

## Fungus

*Saccharomyces cerevisiae*—mitotic  
 recombination  
*Saccharomyces cerevisiae*—reversions  
*Schizosaccharomyces pombe*—forward  
 mutations  
*Saccharomyces cerevisiae*—  
 mitochondrial mutations  
*Neurospora crassa*—ad-3 reversions

## Plant

*Tradescantia*—stamen hair system

## Insect

*Drosophila melanogaster*—sex-linked  
 recessive lethals

Mammal (*in vitro*)

Unscheduled DNA synthesis (human  
 cells)  
 Sister-chromatid exchange (CHO cells)  
 Chromosome aberrations (hamster and  
 rat cells)  
 Specific locus mutations  
 L5178Y cells—TK and HGPRT  
 P388F cells—TK  
 CHO cells—HGPRT  
 Human fibroblasts—HGPRT

Mammal (*in vivo*)

Micronucleus (mouse)  
 Chromosome aberrations  
 Sister-chromatid exchange (mouse,  
 rabbit)  
 Sperm morphology (mouse)

**Nongenetic systems**

Hydroxylation of biphenyl  
 Local greying of hair  
*In vitro* nuclear enlargement  
 Rabin's test  
 Transformation (BHK cells)

# Eukaryotic gene cloning and expression in yeast

from Kim Nasmyth

THE study of the control of gene expression in eukaryotic organisms has been hampered in many cases by the lack of sufficiently powerful genetic procedures to allow conventional genetic analysis of mutants. The recent development of recombinant DNA technology, providing the ability to produce large amounts of individual DNA sequences in pure form, has stimulated an alternative approach, that of the direct study of the DNA sequences constituting the gene without recourse to the phenotype. Although this approach has yielded remarkable insights into the structure of eukaryotic genes the problem of studying their control remains. Ways are needed in which the expression of eukaryotic genes, isolated as cloned recombinant plasmids or phages can be studied in an isolated and manipulable environment. Ideally, once a gene has been cloned in a system in which it can also be expressed, it should be possible to identify not only structural but also control sequences (such as promoters) by *in vitro* alteration of the DNA sequence followed by its reintroduction into the cloning host, with subsequent testing of its continued ability to function. Such a procedure would be an *in vitro* counterpart to the use of mutants in conventional genetic analyses (that is, if you can't find them, make them!).

**λ vectors**

A start in this direction has recently been made by K. Struhl and R. W. Davis (Stanford University) on the expression of the yeast *his3* gene cloned in phage λ. This gene, which codes for IGP dehydratase, is fully expressed in *E. coli*; indeed, it was originally isolated by virtue of its ability to complement *hisB* mutants of *E. coli*. Moreover, its expression can be directed not only by the powerful λ promoter but also by one located in the yeast DNA, the position of which was the subject of investigation. One advantage of λ over plasmids for this type of work is that it is easy to select for mutants with deletions in the inserted DNA, each of which may be tested for their continued ability to complement the *hisB* mutants. To ensure that such deletions do not actually enter, and therefore destroy, the structural gene, they can also be tested for expression using the λ promoter. In this way, Struhl and Davis have identified a pro-

motor region very close to, but not including, the 5' end of the gene that is necessary for its transcription in *E. coli*.

Unfortunately, this particular approach is unlikely to be generally applicable as most eukaryotic genes cloned so far, with the exception of several from yeast, do not seem to be expressed in *E. coli*. Even if they were, it is uncertain whether their control would be particularly relevant. What is needed is a system in which cloned genes can be tested for function in an environment more similar to that of their natural host. One such system is the *Xenopus* oocyte, which is already well established for the study of mRNA expression, and has recently also been used to express recombinant DNA containing 5S rRNA or tRNA genes. However, this system only allows temporary expression. A useful alternative would be a eukaryotic organism into which exogenous genes could be inserted, grown, and manipulated as is now possible in *E. coli*.

The budding yeast *S. cerevisiae* is an obvious candidate for a eukaryotic cloning vehicle. It is easy to handle, its genetical procedures are extremely powerful, its DNA has a complexity only three times that of *E. coli* so that many of its genes have already been isolated by present cloning procedures, and it even contains a closed circular plasmid suitable as a potential vector. However, the crucial ingredient for a cloning system, the ability to transform the host cell's phenotype through the introduction of exogenous DNA, has until now eluded yeast geneticists. The trouble is that yeast possesses an extremely tough cell wall which renders it difficult enough to extract intact cellular constituents let alone introduce them.

**Bacterial plasmid vectors**

With the cloning in *E. coli* of yeast genes for which there are stable mutants, this last bastion has fallen surprisingly quickly (Hinnen, Hicks & Fink *Proc. natn. Acad. Sci. U.S.A.* **75**, 1929-1933; 1978). Hinnen *et al.* treated spheroplasts (cells stripped of their cell wall by enzymatic means) of a non-reverting double mutant of *leu2* with calcium, polyethylene glycol (which promotes cell fusion), and a ColE1 plasmid containing the wild type yeast *leu2* gene (pYe*leu10*). They then regenerated them in a solid medium unsupplemented with leucine and found that a very low proportion of the surviving cells (10<sup>-7</sup>) had become

tween the two studies 12 separate evaluations of the Ames assay will be made, allowing an evaluation of its interlaboratory reproducibility.

Apart from assessing individual assays, the study might also indicate where tests duplicate each other and where they make unique contributions. The study is already fully subscribed. It is coordinated by Drs P. Brookes, B. A. Bridges, I. F. H. Purchase, and J. Ashby in the UK, F. J. de Serres in the US, and T. Sigimura in Japan; and is being conducted under the auspices of the International Association of Environmental Mutagen Societies.

The initial appraisal is planned for the summer of 1979; non-participant enquiries may be directed to Dr J. Ashby, ICI Central Toxicology Laboratory, Alderley Park, Cheshire, UK, or Dr F. J. de Serres, NIEHS, Research Triangle Park, North Carolina. □

Unless otherwise stated, the source of all results quoted in this article was the recent 9th International Conference on Yeast Genetics and Molecular Biology, held in June, 1978 at Rochester, New York.

prototrophic. Tetrad analysis in conjunction with Southern blotting revealed that transformation had mainly occurred through the integration of the entire hybrid plasmid at homologous chromosomal sites (principally at the *leu2* locus, although also at other sites which contain a repeat sequence present on the plasmid). Furthermore, the integrated hybrid plasmid could be recovered intact by excision with a restriction endonuclease followed by transformation of colicin E sensitive *E. coli*. Thus, this system could, in theory, be exploited as a primitive cloning vehicle. In fact, its present value as such is limited not only by the low frequency of transformation obtained but also because the introduced DNA might be altered in the process of integration. Such is the pace of research in this field that both of these problems have already been elegantly solved, with the result that at least two types of versatile vector may very shortly be on the market.

The first of these stems from the observation that the frequency of transformation of different loci by similar *E. coli* plasmids containing different inserts varies considerably. For instance, J. Carbon (University of California) has found that while plasmids containing *leu2* or *his3* gave frequencies in the order of  $10^{-6}$  to  $10^{-7}$ , those containing *arg4* or *trp1* yielded transformants in the order of  $10^{-4}$ . These differences have been more thoroughly investigated and exploited by Davis's group. They found that the plasmid pBR322 containing *his3* gave a low level of transformants, in all of which the plasmid had been integrated at the *his3* locus. However, if a fragment containing *trp1* was added, then the frequency of *his* transformants was increased by a factor of  $10^2$  or  $10^3$  (all of them were also transformed for *trp*). Surprisingly, the introduced plasmid was not integrated but present as a closed circular molecule, at a concentration of approximately one per cell. The tentative explanation for this phenomenon is that the *trp1*, but not the *his3*, fragment possesses a DNA replication origin. It would seem that the replication of the *his3* fragment (and the bacterial plasmid sequence joined to it) is conditional on integration, whereas any hybrid containing the *trp1* fragment can replicate autonomously. However, there is at present no independent evidence that specific replication origins exist in yeast or indeed in other eukaryotic organisms.

The initial interest in *trp1* stemmed from its proximity to the centromere of chromosome IV. It is not yet known

whether a centromere is present on the plasmid nor, if it is, whether it is important for the plasmid's autonomy and stability. If it is active, then the plasmid may be behaving as a minichromosome. Irrespective of the mechanism, this phenomenon can be usefully exploited. A similar *trp1* fragment has been inserted into  $\lambda$  to form a hybrid that will also replicate as an autonomous closed circular molecule once introduced into yeast. Thus, a new breed of hybrid vector has been obtained that will not only transform *trp* yeast strains at a high frequency but will also form viable plaques (even from crude lysates of yeast) in *E. coli*. It is Davis's intention to replace the repressor region of  $\lambda$  by the *trp1* fragment, thereby freeing its inessential central region for cloning, with all its advantages for insert selection.

#### Yeast 2 $\mu$ m plasmid

The second type of vector has been developed using the yeast 2  $\mu$ m plasmid. This molecule is found in nearly all strains, at 50 to 100 copies per cell. Although there is continuing debate as to whether it is located in the cytoplasm or in the nucleus, it is known that its replication is subject to the same controls as those for nuclear DNA (Livingston *Genetics* 86, 73; 1977) and is confined to the beginning of S phase (V. Zakian & W. Fangman, University of Washington). Several transcription products from the plasmid have been identified (J. Broach, C. McGill & J. Atkins, Cold Spring Harbor Laboratory) but there is no evidence for its function. For instance, no genes have been conclusively linked to it. Despite this ignorance, several groups (Davis; G. R. Fink, Cornell University; J. D. Beggs, Plant Breeding Institute, Cambridge) (*Nature* in the press) have constructed, in *E. coli*, hybrid molecules composed of the yeast plasmid, an *E. coli* plasmid, and a wild type yeast gene such as *his3* or *leu2*. Such mongrels will transform appropriate yeast auxotrophs at a very high frequency; in one case as high as  $3 \times 10^{-3}$ . The transformants possess multiple free copies of the introduced plasmid and, in some cases at least, also a single integrated copy. The state of prototrophy of all transformants is inherited in a non-Mendelian manner (as is the resident plasmid) although with different degrees of both mitotic and meiotic stability depending on the type of composite plasmid. Here too, the transforming plasmid can be easily recovered in the appropriate strains of *E. coli* by selecting for either *E. coli* plasmid-specified antibiotic resistance or yeast DNA-specified prototrophy after transformation with yeast ex-

tracts. In its present form, the vector is still in its infancy, but may soon be streamlined by removing all sequences other than those strictly necessary as a marker for transformation of both *E. coli* and yeast (for example the yeast *leu2* gene) and the replication origins necessary for growth in both organisms.

The outstanding features of both the *trp1* and the 2  $\mu$ m plasmid based vectors are that they can just as readily be grown in *E. coli* as in yeast, thereby retaining the different opportunities that each offers; principally, the use of *E. coli* to construct gene banks and to amplify sequences, and yeast to study gene function. Possibly, both types of hybrid vector reside in the nucleus whilst in yeast, thus enjoying an appropriate environment for the study of gene control. One can envisage one possible drawback to the use of these vectors. This is that due to their wider host range, which may indeed extend to other eukaryotes, they may be considered as a greater biohazard than present vectors.

The immediate implications of this work will of course mainly concern yeast geneticists. Given the high rates of transformation, one may now confidently select for any yeast gene for which there is a mutant to be complemented. There are many interesting genetic loci, such as the mating type locus and those involved in the cell cycle and meiosis, for which no gene products, let alone a hybridisation probe, are presently assigned or available. Their DNA sequence may now be directly obtained. For such loci, it is going to become, ironically, much easier to isolate their genes and study the control of their expression than to understand the function of their gene products.

In the longer term lies the potential for using yeast vectors for studying the expression of genes from higher eukaryotes. It remains to be seen whether such genes will indeed function in the yeast environment. There is hope as far as transcription is concerned, for yeast possesses a typically eukaryotic spectrum of RNA polymerases. On the other hand, it is less certain whether

#### Erratum

In the article 'Lunar atmosphere past and present' (*News and Views* 273, 489; 1978) the last sentence in paragraph 2 of column 3 should read "A crater 1280 m across and 180 m deep", and not "... 128 m across ...". In paragraph 3, column 3, line 16 should read "... "It is highly unlikely that the mass distribution of the cosmic dust cloud has changed considerably in the past 10<sup>9</sup> yr," and not "... "10 yr ...".

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correct processing will occur. The proportion of yeast genes that function when cloned in *E. coli* is surprisingly high, suggesting that, unlike higher organisms, many, or indeed most, are not 'split'. If they are expressed, then for some, at least, the complementation of yeast mutants may constitute a painless means of their isolation. □

## France leads in vitrification of radiowastes

from Robert W. Cahn

It has been clear for more than a decade that the most acceptable known way to store highly radioactive wastes over the long term is to incorporate them in glass blocks which are then buried (the 'vitrification' process). The recent proposal, made severally by Ringwood in Australia and Roy in America, to incorporate wastes in synthetic rock may prove one day to be an acceptable alternative. Whichever (glass or 'synroc') wins greater acceptance in due course, such blocks will eventually need to be buried in appropriate geological formations, and a very extensive, systematic assessment of such formations in the UK is now in progress (Chapman, *Gray & Mather Atom*, No. 261, 185, July 1978).

A captious critic might comment that large-scale vitrification has been a gleam in the eyes of a number of scientific fathers for a long time, but that conception does not seem to be followed by gestation let alone parturition; meanwhile, concentrated radioactive solutions are fast accumulating. All the more credit, therefore, to the French Atomic Energy Commission which on 26 June this year inaugurated a production facility at Marcoule, in the Rhone Valley, to vitrify all the waste currently reprocessed at Marcoule, in addition to dealing with accumulated liquid wastes over the next 10 years.

An account of the investigations which preceded this event was given by E. Sombret on 28 June at a colloquium held at Saclay, the French nuclear research centre. All research has been concentrated on aluminoborosilicate glasses based on  $\text{SiO}_2$ ,  $\text{B}_2\text{O}_3$  and  $\text{Al}_2\text{O}_3$ , and a considerable range of compositions has been assessed. The proportion of solid waste incorporated in the glass depends upon the reactor type from which the spent fuel came; light-water reactor wastes,

for instance, make up 24% by weight of the glass, and this implies a volume reduction by a factor of 7.5 times from the original aqueous concentrate, itself of course less voluminous than the original fuel before reprocessing. (A current Swedish programme, outlined in *Swedish Nuclear News* for June 1978, to encapsulate entire spent fuel elements in highly impermeable artificial sapphire by means of a high pressure technique at which the Swedes are adept, will be favoured by opponents of reprocessing, but implies very large volumes for subterranean storage.)

Extensive long-term tests were carried out on the stability of the glass blocks towards leaching by water at ambient temperature. The leaching rate stabilised after a month, at a rate which (to judge from figures quoted by Sombret) would be equivalent to the release of about one  $\mu\text{Ci}$  of activity per day from a 2 kg block containing some hundreds of curies of activity. Large doses of  $\beta$ -radiation did not measurably affect this rate of leaching. Thermal stability was also tested: devitrification takes place at measurable rates above  $\sim 600^\circ\text{C}$ . Partially devitrified glasses did not leach faster than fully vitreous blocks.

To test the possible effects of  $\alpha$ -radiation and concomitant injection of helium into the blocks, glasses artificially enriched in the actinides Pu, Am and Cm were made up and tested over a prolonged period. No detectable changes in mechanical properties were encountered, neither was the leaching rate increased. This finding is important because actinides are much longer-lived than the fission products. A further programme of research on the effects of the presence of  $\alpha$ -emitters is to be initiated.

In designing optimum glass compositions, several mutually conflicting requirements—ease of melting, ability to dissolve in the glassy structure all the fission products and actinides from a particular reactor type—led to a range of different compositions for the various types of waste. On present indications, based on over 10 years of investigation, the stability of these glasses appears very satisfactory (even if a recent commercial advertisement, offering glass-melting furnaces for sale, guarantees the stability of the firm's glasses for a million years, is perhaps a little ingenuous. Which executive will undertake to be around in 1,001, 978 AD to make good the guarantee?)

From 1969 to 1973, the French CEA operated a batch vitrification plant at Marcoule, and prepared 12 tonnes of glass with up to 3000 Ci  $\text{l}^{-1}$  of glass. Powdered glass stock and a stream of radioactive solution were slowly melted together and blocks then

cast. A large-scale continuous plant, the AVM, was designed on the basis of satisfactory results with the batch plant. The cast blocks are to be force-cooled by circulating air for some years; thereafter, activity will have sufficiently decayed for cooling by natural convection to suffice (and at this point the blocks will be ready for geological disposal). Even if forced ventilation should break down, the research results indicate that leaching of the blocks will not be unacceptably accelerated. A further plant for La Hague is planned to be ready in 1982/3, sufficient to vitrify all current wastes from French light-water nuclear power stations.

The 1978 CEA Report claims that French experience in this field is encountering great foreign interest and expresses the hope that the technology will be internationally adopted.

It is conceivable that further improvements may stem from current research on the dense glasses used for the windows of remote-handling cells. In a recent publication of the German glass-maker, Schott, the role of ceria additions of 0.5–2.0% in stabilising such glasses against progressive loss of transparency is outlined. This form of stabilisation might have implications for enhancing the stability of vitrified wastes to chemical changes well below the devitrification temperature. □

## The ribosome: still a knotty problem

from Knud H. Nierhaus

ribosomology has come a long way since the first EMBO workshop 10 years ago when even the number of components of the *Escherichia coli* ribosome had not been settled. Now, a rapidly increasing body of knowledge of the primary structure of ribosomal proteins and RNA means that the ribosome will be the first organelle of which the total sequences of its components will be known in the foreseeable future.

Recent developments in the rapid sequencing of nucleic acids in particular have stimulated a sequence explosion. The complete sequence of the 16S RNA was shown by H. Noller (University of California, Santa Cruz), which is an improvement in many details over the sequence data of the Strasbourg group. Y. Kaziro (University of Tokyo) compared the primary sequence of a part of the elongation factor EF-Tu, derived from the DNA sequence of the *tufA* gene on the one

\*The sixth EMBO workshop on ribosomes, organised by D. Vázquez (Madrid), took place in Salamanca, Spain, on 26 June–1 July, 1978.

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hand and the amino acid sequence on the other. The most spectacular news in this field came indirectly from M. Nomura's group at the University of Wisconsin. They have sequenced large DNA fragments from both clusters of ribosomal proteins (encoded by the *strA* and *rif* regions of *E. coli*) providing detailed information on promoter structure, organisation of transcriptional units, and the amino acid sequences of the proteins. Clearly, the rapid DNA sequencing techniques will change the strategy for determining protein sequences, and it will be interesting to compare the DNA sequences with the more than 40 *E. coli* proteins sequenced so far by conventional techniques (B. Wittmann-Liebold, Max-Planck-Institut, Berlin).

One welcome announcement was that a common nomenclature for eukaryotic ribosomal proteins has been agreed under the coordination of E. H. McConkey (University of Colorado, Boulder). Some details of eukaryotic ribosome structure have been elucidated using established techniques, and the first antigenic determinants from the proteins S1, S2 and S16 have been mapped on the surface of the 40S subunit using immunoelectron microscopy (F. Noll, Akademie der Wissenschaften, Berlin-Buch). Despite differences in size, the subunits of bacterial and eukaryotic ribosomes are very similar by electron microscopy (J. Lake, University of California, Los Angeles).

The secondary and tertiary structure of RNA is now becoming accessible through various new techniques. For 16S rRNA C. R. Cantor (Columbia University, New York) has shown, by psoralen cross-linking which links the bases of adjacent RNA chains, and subsequent analysis of the resulting RNA loops in the electron microscope, that the RNA skeleton of the ribosome may be 'knotted', and base pairing probably occurs between RNA regions far away from each other on the extended RNA thread.

The changes in tRNA conformation on codon-anticodon interactions were discussed at a companion workshop on tRNA structure. With the established technique of kethoxal modification (which modifies specifically G in single strands) R. Wagner (Max-Planck-Institut, Berlin) found an 'opening' of the tRNA structure upon codon-anticodon interactions. Just the opposite was reported by M. Sprinzl (Max-Planck-Institut, Göttingen) who mimicked the codon-anticodon interaction by the anticodon-anticodon

interaction of tRNA<sup>Glu</sup> and tRNA<sup>Phe</sup> and analysed the NMR spectra and the melting temperature. The interaction led to an enhanced stacking and rigidity but no gross conformational change or opening of tertiary structure. The two model reactions are possibly not comparable, and it is not clear whether even one of the approaches reflects the tRNA structure properly, after codon-anticodon interaction on the ribosome.

Another aspect of tRNA interactions at the ribosome was treated in a model proposed by C. Woese several years ago. This model predicted a stacking shift in the anticodon loop when the tRNA is translocated from the A-site to the P-site. Various experiments were reported which did not support the Woese model. A tRNA<sup>Phe</sup> with an aminoanthracene at the Y-base position showed the same fluorescence signals at both sites which is inconsistent with a stacking shift in the anticodon loop (B. Hardesty, University of Texas, Austin). By means of irradiation E. Kuechler (University of Vienna) could crosslink the Y-base of Phe-tRNA<sup>Phe</sup> to poly(U) equally well in the A- and the P-site. This indicates a similar orientation between the Y-base and poly(U) stretch in both sites which is also inconsistent with the Woese model.

The genetic mapping of the ribosomal genes in bacteria is nearly complete. A. Bollen (University of Bruxelles) and K. Isono (Max-Planck-Institut, Berlin) surveyed the state of the art for rRNA and r-protein genes, respectively. Six of the seven known genes for rRNA in *E. coli* have been mapped. The genetic loci of all the ribosomal proteins except six (S1, S9, L13, L20, L31, and L34) have been identified. In addition to the well-known clusters at 72 min (*strA*) and 88 min (*rif*) a third cluster may be present at 95 min where the three proteins S6, S18 and L9 have been found. In summary, the ribosomal proteins and their modifying enzymes are located at at least 12 different positions on the *E. coli* chromosome.

An interesting example of probable regulation at the translational level was reported by S. Pederson (University of Aarhus). He found that the rifampicin-induced increase in the amount of the  $\alpha$ -subunit from RNA polymerase was not paralleled by an induction of S4 and L17 synthesis although these proteins were reported to be present on the same transcriptional unit. During the induction period the amount of mRNA for the  $\alpha$ -subunit is not increased.

The binding of the factors IF-2, EF-Tu, and EF-G to the ribosome is mediated or at least stabilised by the ribosomal protein L7/L12, which is the only protein present in multiple copies

on the ribosome. Each of these complexes (ribosome·factor) shows GTPase activity, and a feature of the GTP cleavage is the efficient and quick dissociation of the respective factor. At least in the case of EF-Tu the GTPase centre seems to be located on the factor, since this factor complexed with the antibiotic kirromycin exerts GTPase activity. However, D. Donner (University of Uppsala) reported that the complex L7/L12·EF-Tu itself has high GTPase activity. Furthermore, a polypeptide which seems to be a C-terminal fragment of L7/L12 showed ATPase and GTPase activities indicating that possibly L7/L12 together with EF-Tu carries the active centre. Strangely enough, this fragment could be isolated from the ribosome only and not from purified L7/L12. In contrast, A. Parmeggiani (Ecole Polytechnique, Palaiseau) could not find GTPase activity on mixing L7/L12 and EF-Tu.

In spite of progress both on the structure and function of ribosomes, many questions remain open: The elucidation of the quaternary structure is still only just beginning, practically nothing is known on the mechanism of translocation (that is, the relative movement of the codon-anticodon linked mRNA-tRNA complex and the ribosome), and we are far from an understanding of even the gross structure-function relationships. □



### A hundred years ago

WE are glad to see that the *Times* is beginning to recognise the national importance of science-teaching in schools, and the necessity for our legislators being able to estimate the bearings of the various problems in physical science which are involved in the measures that come before them, in which the national welfare is involved.

From another leader on Prof. Huxley's address we may infer that this chief representative of average British opinion has advanced from its obstinate opposition to the doctrines associated with the name of Darwin; indeed the tone of the article to which we refer seems even more "advanced" than the address which was the occasion of it. It is comforting to find that, in scientific matters, at least, a better spirit is beginning to pervade the paper which is both an index and a leader of middle-class public opinion.

From *Nature* 18, 22 August, 434; 1878.

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# review article

## Computer chess

Hans J. Berliner\*

*Because of its obvious intellectual content, chess has long presented computer scientists with a challenge to their ingenuity. The development of the attempt to programme computers to play chess at human Grand master level has raised some interesting points about the strategies that men and machines use to solve problems 'intelligently'.*

THE serious possibility of full-scale chess playing by a machine first arose in 1950. The issues at the time were summarised by Claude Shannon<sup>1</sup>, the originator of information theory. He proposed three plausible approaches to the problem, in the form of three different types of program. The first, or type A program depends on what are colloquially known as brute force methods. The program looks at all possible moves in a starting position, and all possible replies to each, for both sides up to a given depth of search. A very simple evaluation function then assigns values to every position at this maximum depth. These terminal values determine what is best play for each side according to the Minimax technique. This method is clearly subject to an exponential explosion of branching possibilities. The amount of work involved in searching a tree of moves is  $B^D$ , where  $B$  (the branching factor) is the average number of alternatives throughout the tree, and  $D$  is the depth of search. Thus each additional layer of depth in the search increases the effort by a factor of  $B$ . For chess,  $B$  is about 35 and even a fast machine cannot provide very many factors of 35 at speed, so depth of search is strictly limited. This led to the proposition of the type B program, which would look at only a subset of moves at any point. This would save the effort involved in examining not only the discarded moves, but all those that sprang from them. The method for discarding moves would be to examine the set of legal moves at a point in the search, and continue searching only those with a certain level of merit as computed by an evaluation function.

Shannon also recognised, however, that humans do not examine chess positions in either of those two ways and he proposed a third type of program—type C—which would play by formulating ideas and investigating their merits until one of them produced a move it liked enough to play. But he did not suggest how this might be done.

This typing of computer programs anticipated some issues of fundamental concern to artificial intelligence (AI). Type A programs depend simply on computational power. Type B programs embody the notion of directing the activity of the machine into more promising areas, obviating the need for quite so much power. Type C is based on the belief that since humans play best, it will be best to simulate their strategies. At present, it

seems that the most effective programs are somewhere between type A and type B; I shall discuss this in detail later.

### History of machine chess

By 1957, one program of each type had been attempted<sup>2-4</sup> but all of them played very badly by human tournament standards. Further, while it was possible to make certain generalisations about the merits of each method, the overwhelming conclusion was that it was very difficult to make a machine play chess well.

The attempt to make a program that plays chess in the style of humans was undertaken by Newell, Simon and Shaw. This team did a great deal of the pioneering work in the early days of AI. Their best program conceived goals and knew methods for trying to achieve such goals. Besides the chess program, they produced other programs that were able to reason and solve problems. The success of these ventures, and the feeling that methods of producing intelligent behaviour were now beginning to be understood, led Simon and Newell in 1957 to make the prediction, among several others, that within 10 years a computer would be World Chess Champion. However, very little work on computer chess was done over the next few years.

In 1966 a type A program named KAISSA (the chess goddess) from the Moscow Institute for Theoretical Physics won a correspondence match with a program at Stanford University by the score of 3-1. However, it was difficult to appraise the capabilities of KAISSA; it was running on a very slow computer, and its play while good at times was inconsistent.

The first major progress in computer chess was made in 1966, when Richard Greenblatt<sup>5</sup> constructed a type B program which soon began to play in human tournaments and was the first program to ever win a game against a tournament level chess player. Within a year of its emergence, it managed to achieve a rating of Class C in human competition. Greenblatt's exploits brought a resurgence of interest to computer chess. His success was based on extremely efficient use of his computer. This meant that more effort was available for searching and analysing of special situations. MacHack VI, as his program was called, was able to deal well with issues such as quiescence: what to do when a position that we would like to evaluate is in such a state of flux that no sensible evaluation is possible. His program could be set to examine a certain number of moves at each depth of the search tree, and this determined approximately how much time it would take on a move.

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This program could play at the human Class C level. The international rating system sets Grandmasters at the highest level, followed by International Masters, National Masters, Experts, Class A, Class B and so on. Thus these programs, while quite capable were still six skill levels removed from the highest calibre of play. However, the fact that until then no program had been able to even achieve the human scale, caused some to believe that a course of steady progress was now beginning. Presumably on the basis of such notions, four computer scientists, Donald Michie of Edinburgh, John McCarthy of Stanford, Seymour Papert of MIT and Ed Kozdrowicki of University of California, made a bet in 1968 for £1,000 with Scottish chess master David Levy that a computer would be able to beat him in a match within 10 years.

By 1970 enough programs were being developed to encourage Monroe Newborn of Columbia University to stage the first US Computer Chess Championship in conjunction with the National Meeting of the Association for Computing Machinery (ACM) in New York city. There were six programs vying for the championship, but unfortunately Greenblatt's was not among them. In its absence the title was won by an amazingly strong type B program from Northwestern University written by three young students, David Slate, Larry Atkin and Keith Gorlen.

The first ACM tournament was a resounding success, drawing a large number of spectators to the special exhibits section of which it was a part. Among the spectators were some of the more notable chess players of New York, who along with the lesser players in the audience guffawed when programs would make obvious errors, or it was announced that the host computer had just broken down, forcing a delay until it could be brought up again. However, the audience appeal was so high that the tournament became an institution at the annual meeting of the ACM. The Northwestern program, initially named CHES 3.0 and now being upgraded each year to versions reaching CHES 4.6, was the perennial winner of this event. Only once did it fail to win when it was upset by a prepared line of play (a tactic from master play the programmers rapidly adapted for their own use).

Progress thrives on competition. During the first few ACM tournaments, a number of programs emerged that were capable of playing a weak tournament level game (Class C to Class D in the American rating system). Programs also emerged in other countries. So, in 1974 at the International Federation of Information Processing Societies (IFIPS) meeting in Stockholm, a Computer Chess World Championship was held. This led to a win by the current version of KAISSA in what was considered an upset when CHES 4.2 was unexpectedly defeated in an early round of the tourney.

By 1975, Greenblatt's program and the Northwestern program had both achieved some results in human tournaments indicating that their play was in the high Class C to low Class B range. However, as an early version of Greenblatt's program had already achieved a Class C rating, it seemed that the current technology had stabilised and another invention was needed to break through toward the goal of Master play and beyond. This portion of the history of computer chess has been extremely well documented by Newborn<sup>6</sup>.

In the meantime, the Northwestern University team had been reexamining the premises upon which their earlier programs were constructed. In 1973, CHES 4.0 became a type A program (one that searches all moves to a given depth). This was done because the type B program occasionally overlooked a good move which had no clear point as seen by the decision function which rejected certain moves; and because some new economies had been found that made the type A search more efficient<sup>7</sup>. This particular change was extremely significant as it combined what is now recognised as the most efficient searching structure with what was already the best chess knowledge structure in any program.

In the fall of 1975, CHES 4.4 made its first appearance on a new super-fast computer (one of the CDC CYBER 170 series). This appearance was another cake walk at the ACM annual

event; however, in the summer of the following year CHES 4.5, little changed from 4.4, won a human Class B swiss-system tournament in California with a perfect score of 5-0. (Swiss-system tournament style allows all competitors to play throughout the tournament in such a way that those with near equal results at any point in the proceedings will play one another. Thus the program here met most of its major challengers.) A few months later it won the Minnesota State Open chess championship (for humans) with a score of 5-1, in the presence of several Experts and Class A players. This was a notable event in that it was the first time a program had ever won an event open to all players, and it achieved a performance rating (based upon the results of its games and the calibre of its opponents) at the Master level. This was enough to attract the attention of even the most sceptical of human observers of the computer chess scene.

It was now clear that the mixture of CHES 4.5's knowledge and the power of the fast computer to aid its searching had created a new level of skill. It remained to see just how good it was. In April of 1977, at Carnegie-Mellon University, CHES 4.5 played a match game with David Levy as part of the aforementioned bet. Although it achieved a very strong position in the early stages, the program allowed the Master to trade down material and entered an unfavourable endgame which it eventually lost. So man was still ahead, but his time was beginning to seem limited.

While Slate worked on new approaches, others took over the task of putting CHES 4.6 through its paces. In an exhibition in New York city, CHES 4.6 played 10 humans simultaneously and convincingly won 8, lost 1 and drew 1 against a field that comprised the former great Internationalist Edward Lasker (who lost) and one or two other players of about Expert strength. Late in 1977, at the Toronto meeting of IFIPS, it decisively won the 2nd World Computer Championship, defeating all its opponents including KAISSA, and thereby making up for its failure at the World Championship three years earlier. A few months ago, Walter Browne the US Chess Champion, played 44 chess opponents simultaneously in Minnesota. Among the opponents was CHES 4.6 which succeeded in defeating Grandmaster Browne. Various earlier versions of the program had also twice defeated Master David Levy when he played several programs simultaneously. In 'blitz chess', played at the average rate of five seconds per move, CHES 4.6 has an excellent record against a collection of Experts and Masters, and has beaten one Grandmaster.

At present all the top programs in the world are type A, although KAISSA is making a break from this structure with a technique called the 'method of analogies' which I shall discuss later. However, while it seems very likely that there will be other Master chess programs in the next few years, only the Northwestern program has any chance of winning the Levy bet for the computer scientists. (At the beginning of the year KAISSA attempted a match with Levy and was beaten rather badly.) From a purely game-theoretical view, the computer scientists made a very bad bet. It is considerably easier not to be beaten than to beat. Thus, winning one game of a two-game match is sufficient to not be beaten, but this would not suffice for the machine; it would also have to avoid losing the other game.

## Knowledge and search

It may seem to some that for a computer to play Class C chess is a mediocre accomplishment. However, such thoughts should be quickly dismissed. For the fastest of modern computers to calculate even 10 moves ahead for each side, considering all possibilities would take 300,000 years. So pure brute force cannot be applied. Further, even a child prodigy could hardly hope to reach the Class C level without at least 1,000 hours of concentrated instruction, study and play. And to achieve Class A would hardly seem possible short of 3,000 hours of such effort.

The basis of human chess strength, by contrast, is accumulated knowledge. It is true that good human players differ



somewhat in their ability to calculate (search along rather narrow, well-defined paths). For instance, former World Chess Champion Mikhail Tahl is well known for his depth of calculation in very complicated positions, where he literally outcalculates his opponents. However, more typical of the excellent player is former World Champion Tigran Petrosian who is better known for his incisive understanding of the positional aspects (those that would take a long time to reveal themselves by merely ploughing ahead) of a situation. However, the calculating ability of even Tahl, as measured by the size of his search tree, is minuscule compared with what a computer manages to look at. The lack of selectivity on the machine's part is due to the fact that computers only avoid searching that part of the search space that can be rejected on mathematical grounds. Human's play, on the other hand, is dominated by ideas; that is, the search pursues an idea until it is found to be satisfactory or is rejected in favour of another.

A good example of the difference in approach to problem solving of man and machine in chess is the following. A good human player will make a particular manoeuvre because he believes that the current position requires it (as indicated by his pattern-recognition capacities), and because he calculates that the manoeuvre cannot be thwarted by his opponent. Thus, he uses prodigious amounts of knowledge in the pattern-recognition process and a small amount of calculation to verify the fact that the proposed solution is good in the present instance. It is not difficult to see how such a facility emerged through evolution; it is capable of extremely sophisticated and rapid response. However, the computer would make the same manoeuvre because it found at the end of a very large search that it was the most advantageous way to proceed out of the hundreds of thousands of possibilities it looked at. CHESS 4.6 has to date made several well known manoeuvres without having the slightest knowledge of the manoeuvre, the conditions for its application, and so on; but only knowing that the end result of the manoeuvre was good.

Thus the performance of the human player is determined by the amount of knowledge he possesses and his ability to use it. With the programs it is also a combination of knowledge and the ability to apply it (accurate search). However, here the search seems to be the dominant factor. This is because chess itself is dominated by considerations of material balance: the desire to capture enemy men while conserving one's own. This type of gain is easy to detect by large searches which need only count the amount of material at the terminus of a search branch. However, this type of search will not pay enough attention to the positional aspects of the game. Thus in situations where material balance is unaffected by best play, a player must do something constructive, if possible, to improve the non-material aspects of his position. Without much knowledge this is very difficult.

There is a definite cost in having knowledge associated with a program. Most obviously, it will require more time to evaluate each terminal node when a complex evaluation procedure exists. Not so obviously, it is extremely difficult to imbue a program with a reasonable amount of non-contradictory knowledge. Humans seem to be able to come to grips with the assimilation of knowledge into a data-base which already contains many facts. No one has yet devised a method for computers to do this in any meaningful domain, so a chess program relies on its programmer for each new item of knowledge. Here there is great difficulty in including a new fact in such a way that it fits in harmoniously with all the existing facts. It should be invocable only at the appropriate times, and should be dominated by certain existing facts, while itself dominating others. Achieving this in a reasonable number of testing steps turns out to be next to impossible.

As long as the memory of a finite state computing machine (even a human being) is in exactly the same state, it will take the same action. However, humans find it much easier to modify their memory in an adaptive way based upon their experiences.

Thus a chess program will play exactly the same game, if its memory state is identical at each point to that in a previously played game. For most programs this condition will be true, as

modifications to their memory are made by the programmers. Thus, if a win against such a program is known, any opponent, no matter what his strength, can choose to repeat the game and win also.

CHESS 4.6 has taken some precautions against this. In the opening, there are situations in which it will choose its move randomly from among several equally adequate alternatives. Later in the game, its move decision will frequently be affected by how much time is available to compute the move. Thus the chances of an identical game are significantly smaller than usual. However, its programmers must still be on guard for any error that it makes in the early stages, as opponents may play deliberately in the hope of reaching the same situation. Thus as the program's play improves, the programmers must be able to display ever greater chess skill to make the correct modifications.

## New methods born of advances in technology

We have now touched upon some of the difficulties in constructing a good program. These difficulties are not inherent only in chess programs; they apply to the development of most programs that purport to be intelligent. Because the basic problems of AI are so well exemplified in computer chess, and because a ready-made scale for evaluating performance exists in the human scale of evaluating chess players' performances, computer chess is considered by many computer scientists to be to AI what the fruit fly was to genetics.

As I have mentioned, human problem solving is based on knowledge. Humans do little searching, and that only to confirm that the approach that they have retrieved from their data-base is nominally correct. But it is possible to trade off knowledge with searching. To see this, consider the difference between the following. (1) Knowing the way to the local bakery. (2) Knowing where it is located. (3) Knowing that there is a bakery within a mile of where we are now. (4) Knowing that there is a bakery somewhere in the city. And (5), just hoping that there is a bakery somewhere in the city. The higher the number of each of these situations, the less knowledge is available and the more searching must be done.

Similarly, programs with more knowledge will need to do less searching than those that have less knowledge. However, the knowledge must be accurate and spread out over useful parts of the domain for this relation to hold. While humans have little difficulty in dealing flexibly with situations such as encountering a barricade while driving down a familiar road, computers must be instructed in each such matter as we have not yet found methods of instructing them to examine their data base to find the applicable techniques. This is the problem which AI is slowly solving. However, along the road there are many possible methods of solving problems in a non-human way, using little knowledge and much search.

That is the case in computer chess. In chess, the extremes of the knowledge-search continuum are patently impossible to cope with: searching all moves to great depths requires aeons of time, and since there are  $10^{44}$  chess positions, storing even a small fraction of these is also out of the question. Thus the computation must use a middle ground approach, but even here a great deal of variability is possible.

Until recently, the computer, with its ability to multiply millions of numbers per second, did not seem to have any unique abilities for solving problems that were so complex that their solution could not be specified in some relatively straightforward (although possibly computationally staggering) way. In the past it had been thought that only toy problems could be solved using methods involving complete enumeration of solutions.

There have been a number of AI programs that are considered to have expert level performance in their domain of competence. The first of these was DENDRAL<sup>8</sup>: a program that

proposed molecular structures for organic molecules. DENDRAL used a great deal of searching to propose and rule out potential molecular structures based upon the laws of chemistry and atomic bonding. Two later programs, MYCIN<sup>9</sup>, which diagnoses bacterial infections and INTERNIST<sup>10</sup>, which diagnoses in internal medicine, both use large quantities of knowledge to achieve their ends. This general trend toward more knowledge-intensive programs has been progressing in AI since its inception and has been considered a necessary part of the advance into more complex domains.

However, computers have become considerably faster over the years, and the increase in machine speed has been accompanied by an increase in understanding of how to get ever more out of them. Further, the ever greater size of immediate memories enables computers to save and compare very large amounts of data that would seem irrelevant to humans. Together, these features make it possible for very large computers to have their own methods of problem solving; methods that differ markedly from those of humans. The implications of this are profound.

One method has been developed by the CHESS 4.6 team. Their program uses prodigious quantities of temporary memory in attempts to find many redundancies in the search and to exploit these to avoid searching certain branches, and to find the best branch as quickly as possible in situations that must be searched. Attention to this type of detail only makes sense in very large searches; humans, who search a maximum of 200 nodes, have no need for such techniques.

Another method has been developed by the programmers of KAISSE<sup>11</sup>. It is called the 'method of analogies' and catalogues certain salient features of positions which have been found during the search to be won for one side or the other. The features and the result are stored in a table, and when a position that matches the essential features is again encountered, it need not be searched because the result is assumed to be known. This method is much closer to that of humans since it is feature dependent; however, the level of abstraction used by the program is far below that which a human player uses.

Recently, at Carnegie-Mellon University two projects for understanding continuous human speech were completed. One used a knowledge-orientated approach involving trying to understand each speech fragment and the role it had in any utterance<sup>12</sup>. The other used a network which gave the *a priori* probabilities that any phoneme would be followed by another phoneme in the particular universe of discourse<sup>13</sup>. It operated by trying to find the best match between what it heard and what could possibly have been said. Despite the fact that an order of magnitude more effort was put into the structural approach, the one using simple *a priori* probabilities turned out to be clearly better. This was because searching through the network to match the utterance proved to be easier and more reliable than coping with great amounts of knowledge. Thus we are now faced with signs that machines may have their own style of problem solving, a style which is alien to us, and which we have to discover. The discovery of this style appears to be very important, as it may be possible to achieve a better level of performance in certain complex but well-defined tasks using this approach than we could using the attempt to mimic the human style.

One point that a chess expert will notice immediately is the tremendous style difference between the play of computers and humans. Since the amount of knowledge in a program is minuscule compared to that which humans of the same standard would command, the machine tends to base its game on a relatively small number of well defined and tested-true concepts. Thus while humans tend to have long-range plans of varying kinds and occasionally make entrepreneurial sacrifices that they feel are promising yet cannot see the end of, a machine plays a stodgy but very accurate game. It will never give up material unless it sees some clear advantage in doing so and likewise will accept all sacrifices for which it can see no serious disadvantage. Any brilliance that it displays will be due to the

fact that it saw to the end of a sequence beginning with an exotic move that an observer thought required great 'insight'.

A searching program's great strength is that it will look at every possibility to some arbitrary depth (3 moves for each side in the case of CHESS 4.6) and then still look at selected lines of play beyond that. Such thoroughness is completely beyond human capacities and even the world's best players will make simple oversights at some time; oversights that a program would be quick to pounce on. Whether this would be enough to make up for the lack of conceptualisation is difficult to tell at the moment. However, the scenario for a machine win is like this: play along until the human makes an error; win some material; swap down material to retain the basic advantage; hope to have enough skill in the endgame to win material advantage. Expert humans, well aware of the scenario, attempt to steer the game into pacific waters where the likelihood of a tactical error is small. They aim gradually to outmanoeuvre the program by having it accede to their wiles, without the program's realising until too late that it has been led down the garden path. However, a critical test will come when the programs have access to additional knowledge. This may allow them to choose plans that deliberately involve great tactical complexities. Then the human may only be able to avoid complex encounters at the cost of allowing his opponent the advantage of not challenging his plan at all. It is not clear whether even a world champion would be able to concede to his machine opponent every risky plan that it started, without drifting into very bad positions.

## Future of computer chess

Being a strong chess player as well as a computer scientist, I find the various predictions/bets rather optimistic on the part of my fellow scientists. However, conjuring up such task deadlines has an excellent effect on the field as it sets targets for the workers to achieve. Thus even if a prediction is wrong or a bet is lost, the field as a whole benefits.

Few humans appear threatened by the fact that we co-exist with a species that can multiply millions of times faster than we can. This may be because multiplying many numbers fast is not really necessary for survival, and can thus be pushed aside as being unimportant. A similar rationale can be applied for dismissing the fact that computers can have almost instant access to billions of catalogued facts. We feel that we have access to these too, only not as fast, so the computer is merely a tool. However, if a computer some time in the future becomes world chess champion, will we have to dismiss chess as an 'unimportant game'? In any case, this will not happen this year, or even in the next five; I consider it possible by 1990.

I do not for one moment believe that there are not tasks that are so complex (such as formulating mathematical systems) that the present day design of computer would be quite outclassed by a human, now and possibly always. But the number of such tasks is diminishing yearly, and chess is on the endangered species list.

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# articles

## The Cerro Galan Caldera, North-west Argentina and its tectonic setting

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*Cerro Galan is the first major caldera to be described from South America, and is one of the largest such structures in the world. Formation of the caldera was preceded by rifting and construction of andesitic composite volcanoes, and followed by further rifting and minor basaltic volcanism. Ignimbrite sheets erupted from the caldera cover over more than 3,500 km<sup>2</sup> and have apparently climbed about 300 m up opposing slopes. The caldera is strikingly similar in morphology, geological evolution and tectonic setting to the classic Valles caldera of New Mexico. There is a clear relationship between block faulting (rifting), construction of ignimbrite shields, caldera formation and alkalic volcanism in both North and South America, which may be related to extensional tectonics in a back arc environment.*

THE Central Andes has long been known as a major province of ignimbrite volcanism<sup>1-3</sup>. Ignimbrite sheets cover enormous areas of the Andean cordillera between latitudes 16° S and 26° S. Individual sheets have volumes of at least 100 km<sup>3</sup> (ref. 4) and can be traced for distances of over 100 km (refs 5, 6). Until the advent of orbital imagery, however, the sources of these large ignimbrites were unknown, mainly because the great size of the source structures precluded their identification from ordinary air photographs. The area of the caldera described here had, in fact, already been visited by geologists who did not recognise its existence. Landsat imagery enabled Kussmaul *et al.*<sup>7</sup> to identify a major caldera on the Bolivia/Argentina frontier. The same structure was also recognised on Spacelab imagery by Friedman and Heiken<sup>8</sup> who also recorded, for the first time, the existence of a large caldera in North-west Argentina. This, the Cerro Galan caldera, was recognised independently by Francis and Baker<sup>6</sup> on Landsat imagery. This article arises from a field reconnaissance of the caldera.

Cerro Galan is located at 67°00' W, 26°00' S in an arid and remote part of the Catamarca province of North-west Argentina. The caldera is elliptical in shape, 40 km by 24 km in size, with its longest axis extending in a north-south direction (Fig. 1a and b). The floor of the caldera is at an altitude of about 4.5 km; the highest points on the caldera wall reach

about 6 km and the resurgent centre reaches a similar height. The Landsat image reveals that the caldera is a relatively youthful feature. Few other calderas in the world attain such huge dimensions, though older examples, such as the Oligocene Emory cauldron in New Mexico, are substantially larger<sup>9</sup>.

### Geological history

A geological history of the area can be outlined. The oldest rocks, which form the pre-volcanic basement, are a series of folded sediments up to 7 km thick, consisting of sandstones, slates and shales of Ordovician age<sup>10,11</sup>, resting on a pre-Cambrian metamorphic basement. The sediments strike north-south, parallel with the major block faults in the area, which are conspicuous on the satellite images. By analogy with the nearby area of the Salar de Cauchari, where Schwab and Lippolt have carried out preliminary geochronological studies, movements of these faults probably commenced before 10 Myr ago<sup>11</sup>, and rapidly led to the formation of a north-south rift valley. The boundary faults of the rift are complex, but can be traced for tens of kilometres north of the area of Fig. 1. Erosion of the walls of the rift led to the partial infilling of the rift valley with sediments.

The rift faults seem to have influenced the location of younger volcanic centres, such as Cerro Cadalaste (Fig. 1), a major composite andesitic cone which is probably about 5 Myr old<sup>12</sup>. Other, pre-caldera volcanic centres are located within the rift valley, one of them on the south-west flanks of the caldera itself. The lavas of these pre-caldera volcanoes rest with strong unconformity on the Palaeozoic basement, or on the rift-infilling sediments.

### Characteristics of the caldera

The location of the caldera on the eastern margin of the rift valley is clear from Fig. 1a and b. Doming and uplift in this area presumably preceded the eruption of ignimbrites from ring fractures and subsequent collapse of the caldera floor along the ring fractures. Ignimbrite sheets spread out radially to cover at least 3,500 km<sup>2</sup>. Individual sheets can be traced for at least 60 km from the centre of the caldera and further work will undoubtedly add to their known extent. Only the western flanks of the caldera were accessible on reconnaissance, but there ignimbrite sheets are spectacularly exposed.

In the outcrops visited, two distinct flow units, each up to 30 m thick, were consistently present, both exhibiting the properties of classic sillar, and the three fold zonation recognised by Sparks<sup>13</sup>. Differences in pumice, lithic and crystal contents were conspicuous between different outcrops, and have still to be systematically described. Columnar jointing is exceptionally well developed in places. Like some other major ignimbrites, the sheets from Cerro Galan exhibit evidence of spectacular mobility<sup>14</sup>, and seem to have descended into the valley between Cerro Galan and Cadalaste to below 4 km altitude and to have climbed up to at least 4.3 km on the slopes of Cerro Cadalaste. This indicates a velocity of at least  $100 \text{ m s}^{-1}$  (ref. 15).

Eruption of the ignimbrites and subsidence of the caldera floor along ring fractures was followed by resurgence of the centre of the caldera and by extrusive volcanism along the ring fractures. Observations from the western slopes, and photo-geological studies show that there are some strong similarities with the Valles caldera of New Mexico. Most of the events described by Smith *et al.*<sup>16</sup> for Valles can be matched with broadly similar sequences at Cerro Galan. In the Valles case, however, an elegant 'necklace' of rhyodacite domes has been constructed along the ring fractures<sup>17</sup>; in Cerro Galan several ill-defined extrusive centres are present along the caldera walls, but there is only one obvious dome, on the south-western margin (Fig. 1).

Resurgence at the centre of the Valles caldera was responsible for some 1 km of uplift<sup>16</sup>, which produced the central Redondo dome, composed mostly of the Bandelier Tuff. The resurgent centre of the Cerro Galan caldera awaits investigation. Photo-geology suggests that it may also be composed of uplifted ignimbrites, but the basement rocks may be exposed there, as in some of the older New Mexico calderas<sup>9</sup>.

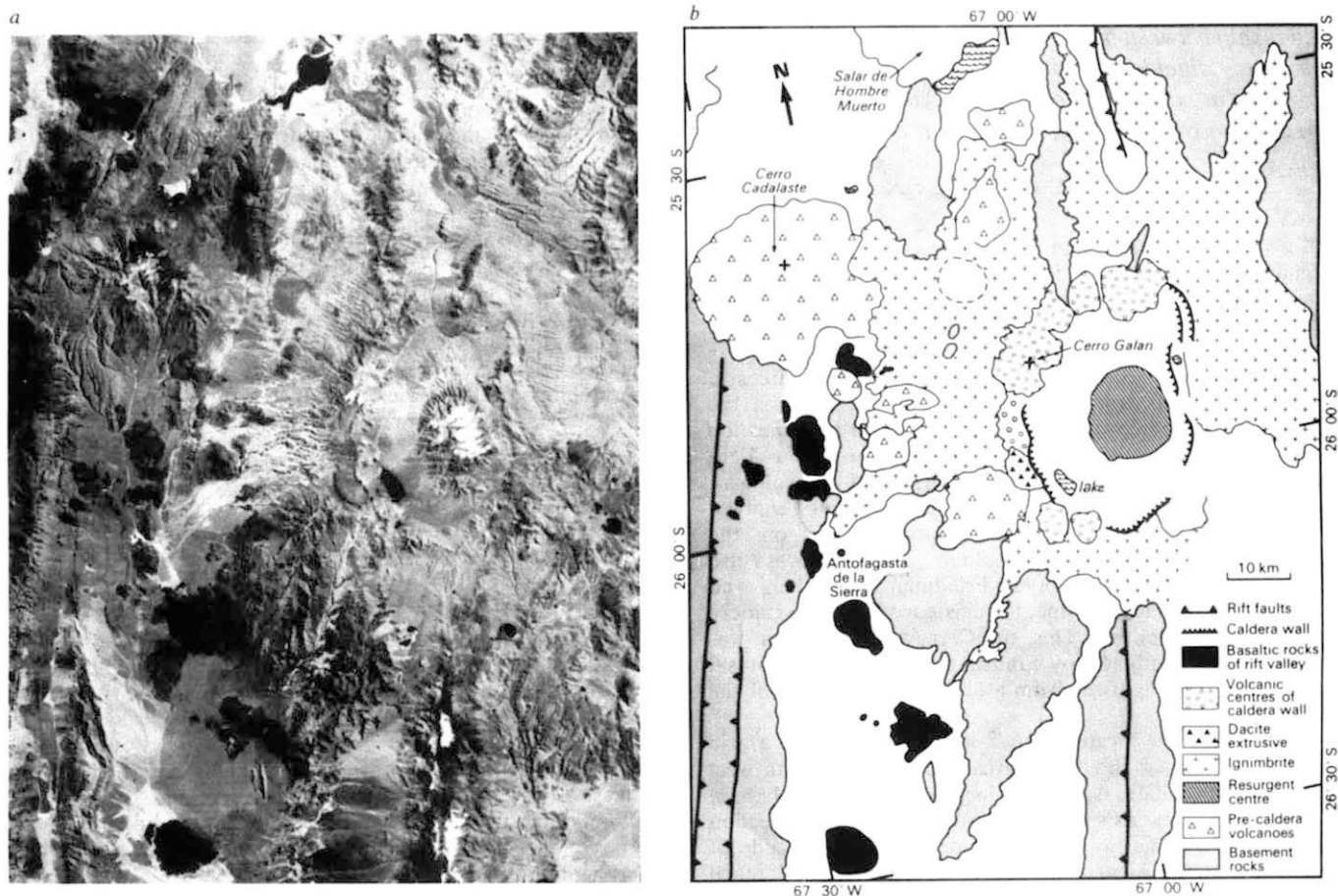
Clarification of this point is necessary before the extent of the resurgent uplift can be determined. A lake exists at present in the south-west part of the caldera floor, and a larger lake probably existed before resurgence took place; therefore, part of the resurgent centre may consist of elevated lake sediments, as in Valles<sup>16</sup>.

## Discussion

The most recent events in the evolution of the caldera seem to have been renewed movement along the rift faults. A fault-controlled valley running north from the caldera originates in a breach in the caldera wall, and extends northwards to become a small graben which displaces the ignimbrite. Away from the caldera itself, the most recent volcanic events were the formation of several extremely youthful basaltic scoria cones on the western margin of the rift, and on the floor of the rift. Two of the latter, 4 km south of Antofagasta de la Sierra, have dammed the drainage of the valley and produced a small lake. Deposits of tufa around the lake indicate that it originally stood many metres higher. Some of these lavas were analysed by Hormann<sup>10</sup> who reported that they were 'latiandesites' rather than true basalts. Other young lavas in the area north of Cerro Galan are more alkalic, and are described by Hormann as 'olivine latites' and 'latites'.

Several other major volcanic structures are now known in the Central Andes, such as Cerro Purico, a previously undescribed structure at latitude  $23^{\circ}30' \text{ S}$  on the Chile/Bolivia frontier. Cerro Galan, however, seems to be unique at present in possessing well defined ring fractures and a resurgent centre, and in its association with rift faulting. The other structures are best described as ignimbrite shield volcanoes, with clusters of andesitic or dacitic extrusives at the centre. Cerro Purico, for

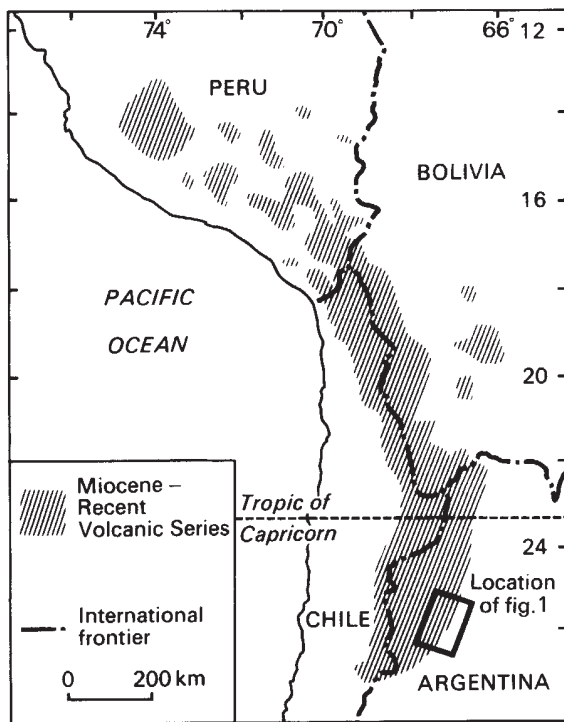
**Fig. 1a** Landsat image of the Cerro Galan Caldera and its surrounding area. The caldera is about 40 km in diameter; the snow-capped peaks reach some 6,000 m altitude. Ignimbrite sheets are well displayed on the northern flanks of the caldera, and are seen as pale-toned, regularly dissected smooth expanses. Centres of recent basaltic activity on the floor of the rift valley show up in dark grey tones. (Landsat MSS band 7, image no82418339350000). **b**, Outline geological map of the area of (a).





example, apparently consists of a complex of extrusive rocks about 20 km in diameter and reaching an altitude of some 6 km which is surrounded by a uniform apron of ignimbrite sheets extending up to 30 km from the centre in all directions.

It has been remarked<sup>18</sup> that there are few calderas in the Central Andes. This is true of 'conventional' calderas a few kilometres in diameter, of which there are only a few examples. It seems that most major Andean ignimbrites are the products of eruptions of shield building volcanoes, rather than the products of plinian eruptions of composite conical volcanoes. The ignimbrite shield volcanoes must rank amongst the largest continental volcanic edifices.



**Fig. 2** The distribution of Miocene–Recent volcanic rocks in the Central Andes. Cerro Galan forms part of a chain of major ignimbrite centres which extends up into Bolivia. These centres are detached from the main volcanic cordillera, and may not be directly related to it.

In the case of Cerro Galan eruption of tens of cubic kilometres of ignimbrites was clearly contemporaneous with the eruption of calc-alkalic andesites and basaltic andesites, and with a more alkalic suite, a situation which exists in other parts of the Central Andes<sup>10,19,20</sup>. It is not easy to see how this association arises. It is sometimes supposed that the basaltic rocks are mantle derived, whereas the calc-alkalic andesites and ignimbrites originate by melting of crustal material at different levels<sup>21</sup>. However, we have argued that the crustal contribution to andesites is minor<sup>19</sup>, and that the dacitic–rhyolitic ignimbrite magmas result from fractional crystallisation of such andesite magmas<sup>20</sup>. In the Mexican volcanic province, both andesites and alkali basalts have similar Sr isotope ratios, and may, therefore, be derived from the same mantle source<sup>22</sup>. Thus, we feel that the source of most of the magmatic material in the Central Andes must lie in the mantle.

## Conclusions

Whatever the explanation, a closely similar situation exists in North America. The Valles caldera is located at the northern extremity of the Rio Grande Rift in New Mexico, within which

small alkali basalt centres are located, and may still be in the process of intrusion<sup>23</sup>. In New Mexico, the magmatism that led to the formation of both the Bandelier Tuff and the alkali basalts was not related to simple subduction zone processes, subduction of the Farallon plate having ceased some 27 Myr ago<sup>24</sup>. Elston has argued that between 35 and 20 Myr ago the southwestern USA experienced a post-subduction 'extensional orogeny', and has suggested that up to 100% extension may have taken place, if the 5–10% extension due to rifting is added to the effects of thinning of lithosphere and pluton intrusion. He further argues that the mid-Tertiary igneous activity beneath the Basin and Range province is analogous to that taking place during back arc spreading, and that the New Zealand and Sumatran ignimbrite provinces lie on the extensions of the Lau and Andaman back arc oceanic basins<sup>25</sup>.

The absence of present day subduction beneath North America makes it necessary to look for mechanisms like those proposed by Elston and others to explain the recent igneous activity there. In South America, however, although Cerro Galan is undoubtedly located in an area of recent extensional tectonics, and is associated with presumably mantle-derived basaltic rocks, it is also located above an active subduction zone dipping at about 25° to the east<sup>26</sup>. It is difficult, therefore, to determine whether the magmatism in the Cerro Galan area is directly related to subduction or to some other less directly related process. On one hand, the smooth variations in andesite composition across the Andean cordillera suggest that these at least belong to a single magmatic suite; only the volumetrically insignificant alkalic rocks stand out as exceptions<sup>20</sup>. This suggests that most of the volcanic rocks in the Central Andes were derived from the subduction zone.

However, Cerro Galan forms part of a major ignimbrite province which extends northwards up into Bolivia, where many major ignimbrite centres are located in the little known Eastern (Frailes) Cordillera (Fig. 2), separated by the 250 km wide altiplano from the volcanic western cordillera<sup>27</sup>. It is difficult to see how these magmatic centres, so widely separated from the active volcanic belt, could be formed by the same subduction zone processes. As the eastern cordilleras of the Central Andes are generally areas of major block faulting and rifting it seems likely that magmatism in these areas may be related to back arc processes of the kind postulated by Elston.

Received 31 May; accepted 19 June 1978.

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# Absolute motion of an individual plate estimated from its ridge and trench boundaries

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*A method is presented for estimating the absolute motion of an individual plate using only the locations of trenches or ridges along the boundary of the plate. The change in trend of the Hawaiian–Emperor seamount chain is shown to be consistent with a chasuring the early Cainozoic in absolute plate motion produced by a change in the location of ridges and trenches along the boundary of the Pacific plate.*

ABSOLUTE motion of plates relative to the mesosphere has been found<sup>1–6</sup> by various methods which require, as input, the known relative motions of all the present plates. However, for earlier times such global analyses are less useful because information is not available for a complete set of plates. Therefore, in making ancient reconstructions it would be useful to be able to estimate the 'absolute' motion of a single plate without requiring information about other plates. We describe such a method here for computing the motion of a single plate and will evaluate its accuracy by comparing the absolute motions found by our method for all the present plates with the motions of the AM1-2 hot-spot model of Minster and Jordan<sup>7</sup>.

## Directions of absolute plate motion

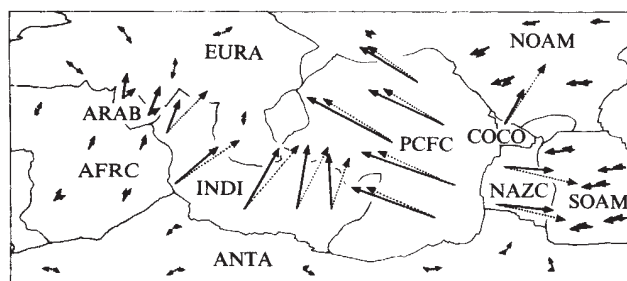
To estimate the direction of absolute plate motion we follow previous workers<sup>3,8–14</sup> in using 'ridge push' and 'slab pull' to drive the plate. We assume that the amount of ridge push per unit length of ridge, and the amount of slab pull are constant and that both of these boundary forces act perpendicularly to the local strike of the ridge or trench. Integration of the torque over a ridge (trench) segment yields a vector parallel and proportional to the chord connecting the endpoints of the segment<sup>3</sup>. Thus we need only know the endpoints of a ridge (trench) segment to evaluate its torque contribution. The ridge push torque vector gives the direction in which a plate would move if ridge push were the only stress acting on a rigid plate. Similarly the slab pull torque vector defines the orientation of an absolute angular velocity vector for a plate acted on solely by slab pull.

For the best estimate of the direction of absolute motion of a plate we equally weight the torque contribution of equal lengths of ridge or trench segment and sum the ridge push and slab pull torque vectors. If the configuration of ridges were known much better than the configuration of trenches for an ancient plate, then we would weight ridges more heavily or vice versa. If different weighting factors were used for the present plate configuration<sup>11–13</sup> the direction of the resultant would not change very much because for most plates the ridge push and slab pull torque vectors are nearly parallel. Our best estimate

of the direction of plate motion is described by an angular velocity unit vector,  $\hat{\mathbf{E}}$ , parallel to the total torque vector.

## Speed of absolute plate motion

The speeds of present plates show a markedly bimodal distribution<sup>4,12</sup> (Fig. 2). We have recalculated mean plate velocities using the relationship  $\mathbf{v} = \omega \hat{\mathbf{E}} \times \mathbf{r}$ , where  $\mathbf{v}$  is the velocity at a point with position vector  $\mathbf{r}$ ,  $\hat{\mathbf{E}}$  is the angular velocity unit vector, and  $\omega$  is the rate of rotation. Using values of  $\hat{\mathbf{E}}$  and  $\omega$  from Minster and Jordan<sup>7</sup>, we found the mean velocity,  $\bar{v} = \omega |\hat{\mathbf{E}} \times \mathbf{r}|$ , where  $|\hat{\mathbf{E}} \times \mathbf{r}|$  is the scalar length of  $\hat{\mathbf{E}} \times \mathbf{r}$  averaged over the surface of each plate. Oceanic plates attached to sinking slabs (including the hybrid Australia–Indian plate) have a mean velocity of  $\bar{v} = 82 \text{ mm yr}^{-1}$  whereas continental plates with

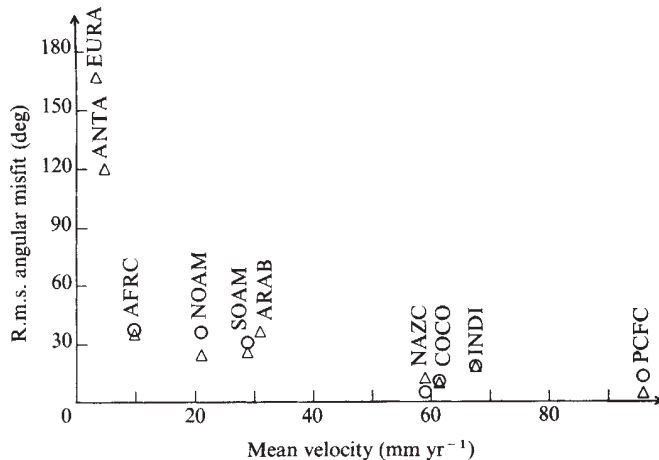


**Fig. 1** Absolute plate velocities at selected points. Solid arrows, velocities calculated from the AM1-2 model of Minster and Jordan<sup>7</sup>. Dotted arrows, velocities estimated by our individual plate boundary method, in which oceanic plates are assumed to travel at a mean velocity of  $82 \text{ mm yr}^{-1}$  and continental plates travel at a mean velocity of  $14 \text{ mm yr}^{-1}$ . The hybrid Australia–Indian plate has been lumped with the oceanic plates because of its large downgoing slab. The plate boundaries shown are adapted from Solomon and Sleep<sup>3</sup>.

little or no downgoing slab have a mean velocity  $\bar{v} = 14 \text{ mm yr}^{-1}$ . It seems reasonable, therefore, to begin with a first-order model in which all oceanic plates with attached slabs have mean speeds of  $82 \text{ mm yr}^{-1}$  and all continental plates with little or no slab attached have mean speeds of  $14 \text{ mm yr}^{-1}$ . As a physical basis for this model, Forsythe and Uyeda<sup>12</sup> suggested that the oceanic plates move at a uniform velocity because the sinking slab reaches a terminal velocity. Alternatively, Harper<sup>11</sup> has suggested that while drag under plates is important, plates with less plate area tend to have ridges closer to trenches, and, therefore, the downgoing slab is warmer and thinner and, hence, should exert less pull. In our first-order model, we constrain the mean velocity  $\bar{v}$ , to equal  $82 \text{ mm yr}^{-1}$  or  $14 \text{ mm yr}^{-1}$  and solve for  $\omega = \bar{v}/|\hat{\mathbf{E}} \times \mathbf{r}|$ .

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**Fig. 2** R.m.s. misfit in azimuth of the surface velocities between the absolute plate motions calculated in this paper and those of the AM1-2 model of Minster and Jordan<sup>7</sup> averaged over the surface of each plate and plotted as a function of the mean velocity of the plates. ○, Slab pull direction; △, slab pull + ridge push direction.

## Results

The overall fit between our estimates of absolute plate motion and those of AM1-2 is quite good (Fig. 1, Table 1). The fit in direction of plate motions seems to improve with mean plate velocity and is quite good for the oceanic plates, all of which have high velocities. The small r.m.s. misfit in azimuth between our surface velocity vectors and those of AM1-2 of only 11° averaged over the oceanic plates supports this conclusion. For some of the slow plates the two sets of velocities do not agree (Fig. 2). This may be because the ridge push and slab pull forces acting on these plates are comparable in magnitude to the traction exerted by asthenospheric flow and other forces that depend on the motions of neighbouring plates, which we have neglected in our model. However, the errors in the absolute motion vectors for the slowest plates are almost as large<sup>7</sup> as the discrepancy between these vectors and those determined by our method, so the discrepancy may have little physical significance.

For those plates having both downgoing slabs and ridges we calculated plate motions due to ridge push and slab pull acting separately. We found that both these estimates tend to have increasing accuracy with increasing plate velocity (Table 1, Fig. 2) and that their directions are, on average, equally good estimates of the direction of absolute plate motion. Thus, in a reconstruction one could use either ridge push or slab pull to estimate the direction of plate motion. This is useful because only the configuration of one type of boundary, usually a ridge, may be known completely for some ancient plates. Our results show that the location of either the ridges or the trenches along the boundary of one plate provide an adequate basis for estimating the present absolute motion of that plate for all but the slowest plates.

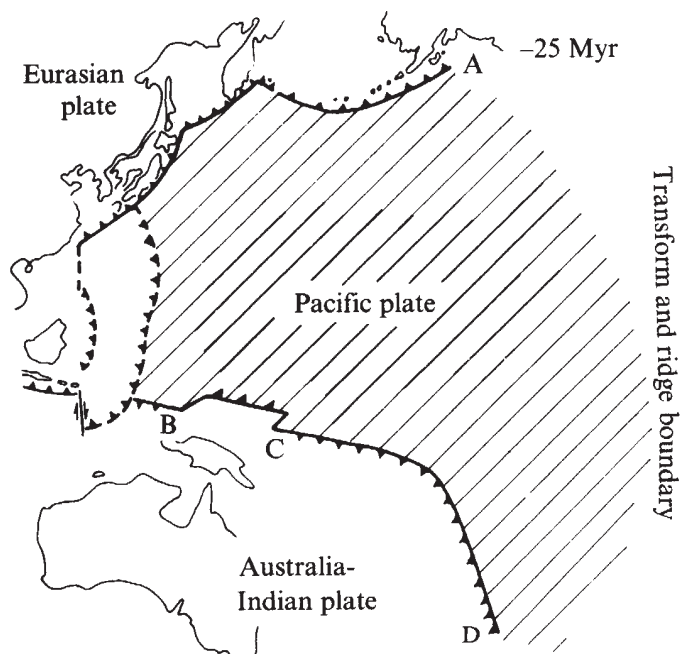
## Application: the Hawaiian–Emperor Ridge

Does the present method work as well for ancient plates? If the bend in the Hawaiian–Emperor seamount chain marks a change in the absolute motion of the Pacific plate 42 Myr ago<sup>15–20</sup>, then this major geological feature is an excellent test. A reconstruction of the Pacific plate at 25 Myr BP by Hilde *et al.*<sup>21</sup> shows a pattern of ridges and trenches not very different from the present configuration (Fig. 3), whereas their reconstruction for 65 Myr BP is markedly different (Fig. 4). We have

estimated the absolute motion of the Pacific plate using the method described above for both of these reconstructions. Our surface velocity vectors agree remarkably well in azimuth with the trends in the Hawaiian–Emperor Ridge before and after the bend and in magnitude with the observed progression of ages along the Hawaiian chain. In the following discussion we have projected all our right hand rule vectors and rotation poles through the origin in order to compare them directly with the left hand rule poles of Clague and Jarrard.

In calculating a slab pull torque vector for the 25 Myr BP reconstruction, we assumed that the Pacific plate was being underthrust all along the sequence of arcuate trenches from point A at the eastern end of the Aleutian trench to point D at the southern end of the Tonga–Kermadec trench (Fig. 3) except for the small segment B–C, corresponding to a region north of southern New Guinea where, until the middle Miocene, the Indian plate was underthrusting the Pacific plate<sup>22</sup>. To estimate the absolute motion of the Pacific plate due to slab pull by our method, we need to know only the coordinates of points A, B, C, and D 25 Myr ago in the absolute reference frame. Point A is marked by a trench–transform junction at the eastern end of the Aleutian trench which we assume has not moved relative to North America during the past 25 Myr. To locate its absolute coordinates at this time we first rotated North America (with point A) to its position at 25 Myr BP relative to the Pacific plate using the North America–Africa–India–Antarctica–Pacific circuit<sup>23</sup> and then rotated both plates along the Hawaiian ridge to their absolute position at 25 Myr BP using the pole of Clague and Jarrard<sup>16</sup>. Points B, C, and D we assume to have moved with the Australia–Indian plate. Small errors are expected in the pole position because of uncertainty in the locations of points B and C and in the extent of late Tertiary deformation along the eastern margin of the plate. The absolute coordinates of these points 25 Myr BP was found by first closing the Australia–Indian plate to the Antarctic plate using the pole of Weissel and Hayes<sup>24</sup>, and assuming the Antarctic plate was, to first order, fixed in the absolute reference frame. The resulting direction of the 25 Myr BP slab pull torque vector has coordinates (72°N, –72°E) relative to

**Fig. 3** Western margin of the Pacific plate at 25 Myr BP, points A, B, C, and D mark the ends of segments of trench of constant polarity. Reconstruction adapted from Hilde *et al.*<sup>21</sup>.



**Table 1** Absolute angular velocities (right hand rule)

| Plate name | A          |       | B         |       | C           |       |          | D     |       |          | E                | F, G, H<br>r.m.s. angular misfit |            |           |
|------------|------------|-------|-----------|-------|-------------|-------|----------|-------|-------|----------|------------------|----------------------------------|------------|-----------|
|            | Ridge push |       | Slab pull |       | Push + pull |       |          | AM1-2 |       |          | Mean plate speed | Push + pull                      | Ridge push | Slab pull |
|            | Lat.       | Long. | Lat.      | Long. | Lat.        | Long. | $\omega$ | Lat.  | Long. | $\omega$ |                  |                                  |            |           |
| PCFC       | -65        | 97    | -48       | 108   | -58         | 103   | 0.82     | -62   | 97    | 0.97     | 96               | 5                                | 3          | 14        |
| INDI       | 36         | 18    | 34        | 43    | 36          | 28    | 0.88     | 19    | 36    | 0.72     | 67               | 17                               | 17         | 20        |
| COCO       | 40         | 168   | 25        | 168   | 33          | 168   | 0.74     | 22    | -116  | 1.42     | 62               | 10                               | 12         | 12        |
| NAZC       | 61         | -48   | 74        | -93   | 68          | -62   | 0.75     | 48    | -94   | 0.58     | 59               | 12                               | 21         | 4         |
| ARAB       | 46         | -68   | —         | —     | 46          | -68   | 0.13     | 27    | -4    | 0.39     | 31               | 36                               | 36         | —         |
| SOAM       | -64        | 142   | -32       | 151   | -63         | 142   | 0.13     | -82   | 76    | 0.28     | 29               | 14                               | 13         | 32        |
| NOAM       | -21        | -44   | -62       | -4    | -25         | -42   | 0.14     | -58   | -41   | 0.25     | 21               | 24                               | 24         | 36        |
| AFRC       | 29         | -77   | 12        | -76   | 26          | -77   | 0.14     | 19    | -22   | 0.14     | 10               | 51                               | 50         | 54        |
| ANTA       | -10        | -165  | —         | —     | -10         | -165  | 0.13     | 22    | 76    | 0.05     | 5                | 120                              | 120        | —         |
| EURA       | 14         | 138   | —         | —     | 14          | 138   | 0.17     | 1     | -23   | 0.04     | 4                | 166                              | 166        | —         |

A, North latitude and east longitude of the direction of the absolute angular velocity vector of a plate acted on only by ridge push. Calculated using the geometrical factors of Forsythe and Uyeda<sup>12</sup>.

B, Similar to A for slab pull.

C, Coordinates and angular velocity,  $\omega$  (deg Myr<sup>-1</sup>), of our absolute angular velocity vector. Location found by summing A and B weighted proportionally to the effective lengths of ridge or trench. The magnitude of the angular velocity vector has been adjusted to produce a mean surface velocity of 82 mm yr<sup>-1</sup> over oceanic plates and 14 mm yr<sup>-1</sup> over continental plates.

D, Coordinates and angular velocity,  $\omega$  (deg Myr<sup>-1</sup>), of the absolute angular velocity vectors of model AM1-2 of Minster and Jordan.

E, Mean plate velocity found from AM1-2 (mm yr<sup>-1</sup>).

F, r.m.s. angular misfit between surface velocities found from our angular velocities (C) and the angular velocities of AM1-2. This value is found by integrating, over the surface of a plate, the squared difference in azimuth (measured in degrees) between the linear velocity vectors for the two angular velocities.

G, Similar to F, comparing the ridge push direction to the AM1-2 angular velocity direction.

H, Similar to F, comparing the slab pull direction to the AM1-2 angular velocity direction.

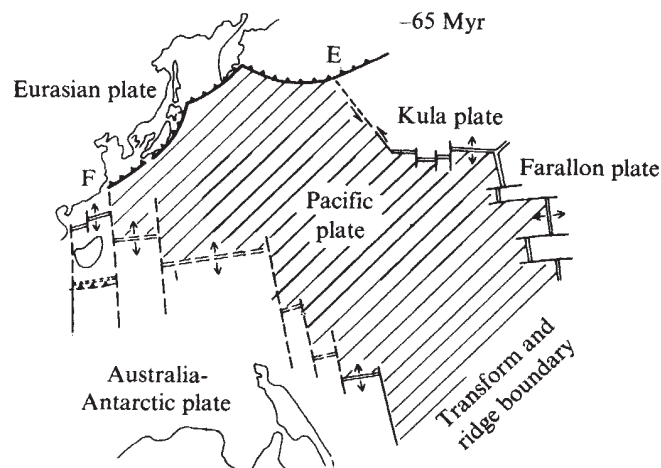
the Pacific plate at that time. Rotating this vector together with the Pacific plate back about the Hawaiian pole to the present reference frame gives the coordinates of (71°N, -73°E), as shown in Fig. 5. As expected, the 25 Myr BP torque vector is not much different from the present one.

The slab pull torque vector for 65 Myr BP was found similarly, using a reconstruction by Hilde *et al.*<sup>21</sup> which shows a trench boundary along only the north and north-west boundaries of the Pacific plate between points E and F (Fig. 4). Following Hilde *et al.*<sup>21</sup>, we assumed that these points have remained fixed relative to Asia which is assumed fixed in the

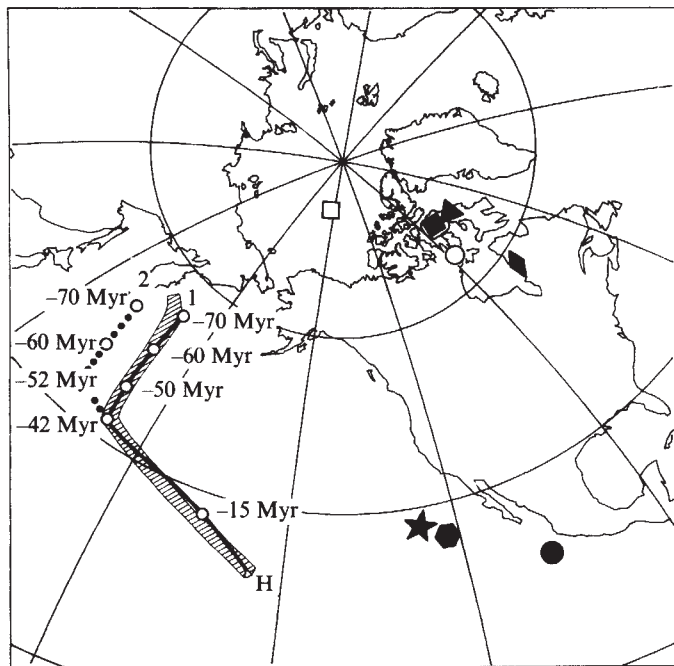
absolute reference frame. The resulting torque vector direction has coordinates (22°N, -98°E) relative to the Pacific plate 65 Myr BP. Rotating this pole together with the Pacific plate 34° about the Hawaiian pole to the present reference frame gives present coordinates for the 65 Myr BP torque vector direction of (27°N, -126°E). The direction of this slab pull torque vector agrees remarkably well (Fig. 5) with the Emperor pole found by Clague and Jarrard<sup>16</sup> from the trend of the Emperor seamount chain. For angular velocity vectors having the orientation of the Emperor pole and 65 Myr BP torque vector, the r.m.s. misfit in azimuth between the corresponding surface velocities is only 15° averaged over the approximate early Tertiary geometry of the Pacific plate. To investigate the dependence of our result on the fixed Asia assumption, we also calculated the direction of this torque vector for a reference frame in which Antarctica is fixed. The location of Asia was found by rotating the Eurasian plate relative to the Antarctic plate using the finite rotation poles used by Solomon *et al.*<sup>20</sup> for 55 Myr BP then extrapolating to 65 Myr BP using their angular velocity vectors. In the present reference frame the direction of this torque vector lies at (24°N, -122°E), which is just a few degrees away from the torque direction calculated for the fixed Asia reference frame and somewhat closer to the direction represented by the Emperor pole of Clague and Jarrard<sup>16</sup>.

We used the angular velocity vectors estimated by our method for 65 Myr BP, 25 Myr BP and the present as approximate finite rotation poles for these times and assumed that they represent the absolute motion of the Pacific plate rve to a Hawaiian hot spot. It is then possible to calculate the location of a model Hawaiian-Emperor Ridge which can be compared with the observed geological feature (Fig. 5). The Hawaiian segment of the ridge can be modelled by two small circle arcs, one centred on the rotation pole parallel to the present torque vector and corresponding to the younger part of the ridge (0-15 Myr BP), and one centred on the Euler pole parallel to the 25 Myr BP torque vector and corresponding to the older

**Fig. 4** Western margin of the Pacific plate at 65 Myr BP, points E and F are the endpoints of the early Tertiary subducting slab of the Pacific plate. Reconstruction adapted from Hilde *et al.*<sup>21</sup>.







**Fig. 5** Tertiary absolute motions of the Pacific plate. Region of diagonal lines extending from H (Hawaii) to near the Kamchatka peninsula is the generalised trace of the Hawaiian-Emperor seamount chain inferred from bathymetry. Curves 1 and 2 correspond to our two models for Pacific plate motion and assume that the bend occurred either at 42 Myr BP (curve 1) or 52 Myr BP (curve 2). Ages along the ridge shown opposite the open circles are ages calculated from our model assuming the mean surface velocity of the Pacific plate was  $82 \text{ mm yr}^{-1}$ . ■, Clague and Jarrard<sup>16</sup> Hawaiian pole; ◆, Current Pacific torque direction; ▲, 25 Myr BP slab pull direction; ●, Clague and Jarrard<sup>16</sup> Emperor pole. ★, 65 Myr BP slab pull direction assuming Asia fixed in the absolute reference frame; ●, 65 Myr BP slab pull direction calculated in the absolute reference frame in which Antarctica is fixed; ○, 55 Myr BP Pacific plate angular velocity of Solomon *et al.*<sup>20</sup> rotated to the present reference frame. □, 55 Myr BP Pacific plate slab pull torque vector for plate boundaries used by Solomon *et al.*<sup>20</sup> rotated to the present reference frame.

part of the Hawaiian Ridge (15–42 Myr BP). Our assumed velocity for oceanic plates of  $82 \text{ mm yr}^{-1}$  corresponds to rotation of the Pacific plate about these angular velocity vectors at a rate of  $0.82 \text{ deg Myr}^{-1}$ . The central angle subtended by the younger arc segment and the magnitude of the corresponding finite rotation is thus  $12^\circ$  and that of the older segment is  $22^\circ$  for a total of  $34^\circ$ , in good agreement with the observed arc length of  $34^\circ$ . The older Emperor Ridge is modelled in Fig. 5 by an Euler pole of rotation parallel to the 65 Myr BP torque vector for the Pacific plate. We found that a scalar angular velocity of  $0.89 \text{ deg Myr}^{-1}$  gave a mean velocity of  $82 \text{ mm yr}^{-1}$  averaged over the approximate early Tertiary geometry of the Pacific plate. The trends of the two ridges and the age of their juncture are represented quite well by the present model.

The explanation suggested by our model for the change in trend at 42 Myr BP of the Hawaiian-Emperor Ridge is the development of trenches along the south-west boundary of the Pacific plate, replacing an earlier set of ridges and transforms (Fig. 4). This new trench system was produced by the north-eastwards motion of the Australia-Indian plate converging with the Pacific plate which was caused by the rifting of Australia from Antarctica. The oldest oceanic magnetic anomalies identified by Weissel and Hayes<sup>24</sup> between Australia and Antarctica have an age of about 52 Myr BP on the

Heirtzler *et al.*<sup>25</sup> time scale; more recent polarity time scales<sup>26,27</sup> would give them a slightly younger age. In our model, the Heirtzler *et al.*<sup>25</sup> age provides a limit for the earliest age of the bend in the Hawaiian-Emperor Ridge (curve 2 in Fig. 5). With slow convergence between the two plates, it is reasonable that 10 Myr would elapse before the subducting slab would be long enough and dense enough to exert significant pull on the Pacific plate and change its direction of motion (curve 1 in Fig. 5).

## Discussion and conclusions

Our analysis of the Hawaiian-Emperor Ridge suggests that several simple generalisations based on the present motion of plates are valid for ancient plate motions. As we have examined only one ancient plate, the agreement of our model with the observed trends of the Hawaiian-Emperor Ridge may be fortuitous. In contrast to our results, Solomon *et al.*<sup>20</sup>, from an analysis of the early Tertiary plate reconstruction of Jurdy and Van der Voo<sup>28</sup>, concluded that many generalisations drawn from present plate motions are not valid for the early Tertiary, and consequently that the early Tertiary motion of the Pacific plate was WNW, nearly parallel to the Hawaiian Ridge and, therefore, the Emperor Ridge could not be the trace of a fixed hot spot.

Part of the disagreement between their result and ours is due to the difference between the early Tertiary reconstructions of Jurdy and Van der Voo<sup>28</sup> and Hilde *et al.*<sup>21</sup>. In the former at 55 Myr BP the trench boundary of the Pacific plate extended down to the Australia-Antarctica plate, whereas in the latter at 65 Myr BP the trench extended only to the latitude of Taiwan. The direction of Pacific plate motion determined by our method from the reconstruction used by Solomon *et al.*<sup>20</sup> is more consistent with the direction of absolute motion they calculated than with the trend of the Emperor Ridge (Fig. 5). The early Tertiary geological history of the south-west boundary of the Pacific plate is an important key to understanding the early Tertiary motion of the plate.

This research was funded by NSF grant EAR76-00120. R. G. was supported by an Amoco Foundation Fellowship. We thank William Dickinson, Robert Simpson, and Eli Silver for their help, also Sean Solomon for the plate boundaries for Fig. 1 and Norman Sleep for a helpful review.

Received 12 February; accepted 31 May 1978.

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# Epidemiological evidence for causal relationship between Epstein-Barr virus and Burkitt's lymphoma from Ugandan prospective study

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*Results from a prospective sero-epidemiological study initiated in Uganda in 1971 indicate that children with high antibody titres to Epstein-Barr virus structural antigens are at high risk of developing Burkitt's lymphoma. These findings strongly support a causal relationship between the Epstein-Barr virus and Burkitt's lymphoma but suggest that the oncogenic potential of the virus is realised only in exceptional circumstances.*

ALTHOUGH there has never been any direct epidemiological evidence that Burkitt's lymphoma (BL) is caused by the Epstein-Barr virus (EBV), there is considerable circumstantial evidence. The virus, first isolated from a biopsy specimen taken from a child with BL<sup>1</sup>, is well known for its ability to transform human B lymphocytes<sup>2,3</sup> and for its *in vivo* oncogenic potential in non-human primates<sup>4,5</sup>. BL patients have been found to have higher levels of antibodies against a wide range of EBV-determined antigens (EBV capsid antigen(s) (VCA), early antigens (EA) and membrane antigens (MA)) than unaffected children of the same age from the same area<sup>6,7</sup>. In addition, multiple copies of viral genomes (EBV/DNA) and EBV-determined nuclear antigen (EBNA) have often been detected

in BL cells<sup>8,9</sup>. On the other hand, BL has a highly restricted geographical distribution<sup>10-12</sup> which led to the suggestion that an arthropod-borne agent might be the cause<sup>11</sup>, whereas EBV is widespread and not known to be arthropod-borne. Nevertheless, by the end of the 1960s the evidence suggesting that EBV was a necessary factor for the development of BL was sufficiently strong to justify a large prospective sero-epidemiological investigation.

## Rationale and design

As infection with EBV is nearly ubiquitous among African children, it was necessary to show that children developing BL were infected with, or reacted to, EBV in a different way from the general population. The difference was to be, for example, in the age at infection, the degree of infection, or the response to infection. It was possible that the EBV serological profile peculiar to BL patients might have arisen as a result of the disease because the proliferating tumour cells could harbour the virus, though elevated EBV antibody titres are not regularly seen in patients with other B-cell lymphomas<sup>13</sup>. To exclude that possibility, serum samples had to be obtained from patients before the onset of disease and this necessitated a large prospective study. A model was provided by the demonstration of EBV as the cause of infectious mononucleosis (IM)<sup>14,15</sup>. Serological tests showed that only students who had avoided EBV infection before entering Yale University were at risk of developing IM. Any association of BL with EBV was likely to be more complex and we formulated four hypotheses<sup>16,17</sup>.

Our first (the null hypothesis) was that there is no causal relationship between EBV and BL. The EBV serological profile of children who will later develop BL should not differ from that of controls matched for age, sex and locality. The high reactivities observed in BL patients after diagnosis would reflect a secondary reactivation of a passenger virus. Our second hypothesis (the IM model) was that BL develops shortly (within 2 yr) after a primary infection by EBV. Sera collected from patients before the incubation period would lack antibodies to EBV. According to our third hypothesis, BL

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develops in children who have had a long and heavy exposure to EBV. The sera of 'future' BL patients would exhibit high antibody titres long before diagnosis of BL. Our fourth hypothesis was that EBV has a causal role in BL, but that the latent periods between infection and clinical onset were long and variable and timely intervention of cofactors was necessary. Before diagnosis the reactivity of the sera from BL patients might differ from that of controls but in an unpredictable manner. This hypothesis could not be tested in a study of reasonable size.

We selected the West Nile District of Uganda (Fig. 1) as the study area as the incidence of BL was high and the epidemiology well documented<sup>18,19</sup>. If either our second or third hypothesis was correct, the study would also yield information about the length of the period between primary infection and onset of disease.

Feasibility studies in 1968 and 1969 showed that within individuals, antibody titres to EBV were quite stable over 18 months<sup>20</sup>. About 10% of children had titres greater than 1/160 and 10% had titres less than 1/10. Assuming that either group (according to the second and third hypotheses) might have a fivefold greater risk than normal, then 30 cases of BL would be needed to ensure a high probability of a positive finding. We estimated that in 5 yr approximately 35 cases would arise in 37,000 4–8-yr-olds living in the study area, which was selected from the most densely populated parts of four adjacent counties (Aringa, Maracha, Terego and Madi (Fig. 1)).

### Organisation of the survey

We explained our purpose at public meetings, and a few days before the collection of blood in each locality we made personal visits and registered all members of each household. Parents brought their children, 2 or 3 d later, to a central point, usually within 2–3 km of their home. More than 85% of eligible children were included in the study in most parishes. However, in Aringa county adequate cooperation was not obtained and so part of Ayivu county was incorporated into the study (Fig. 1).

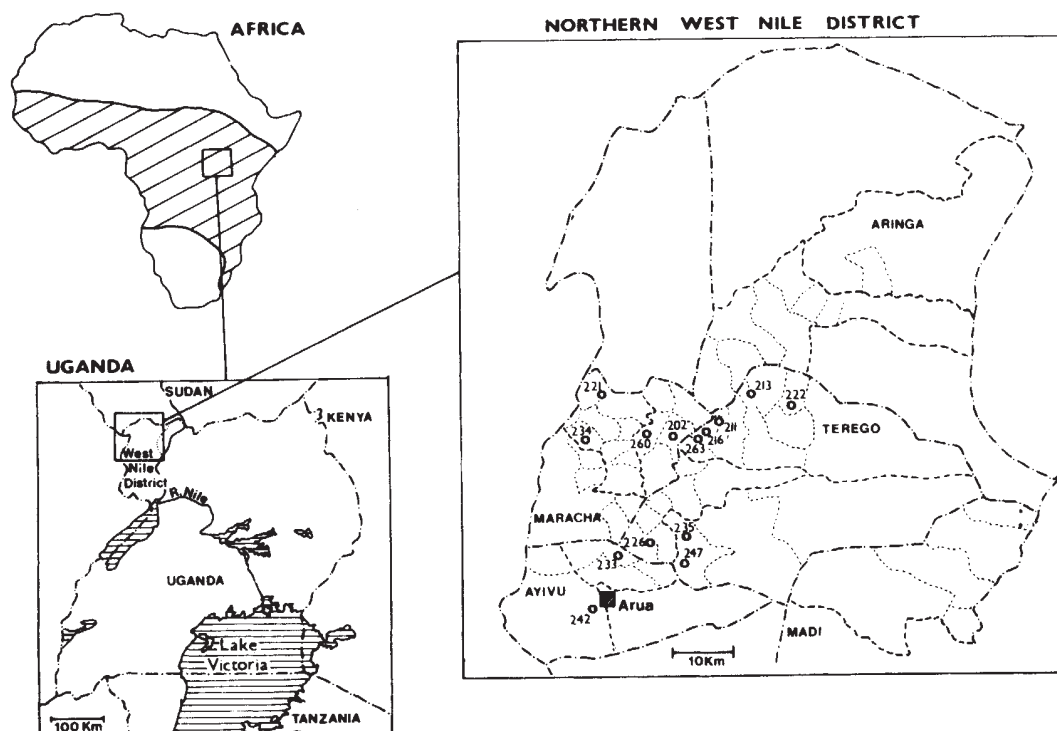
Venous blood was taken whenever possible, but from very young children a finger or heel prick was used. Thick and thin blood films were also obtained for malarial parasite investigation. Serum samples were transferred in insulated boxes

**Table 1** Patients diagnosed with BL in the West Nile District of Uganda in the period 1968–77 by year of diagnosis and county of residence

|      | Aringa, Maracha<br>Terego, Madi | Other<br>counties | Total |
|------|---------------------------------|-------------------|-------|
| 1968 | 7                               | 2                 | 9     |
| 1969 | 12                              | 4                 | 16    |
| 1970 | 7                               | 8                 | 15    |
| 1971 | 10                              | 11                | 21    |
| 1972 | 6                               | 8                 | 14    |
| 1973 | 12                              | 11                | 23    |
| 1974 | 4                               | 2                 | 6     |
| 1975 | 9                               | 9                 | 18    |
| 1976 | 4                               | 5                 | 9     |
| 1977 | 2                               | 5                 | 7     |

containing cooling packs and within 6 h placed in a domestic refrigerator (temperature approximately 4 °C). On the following day, sera were separated and stored in a freezer (at –70 °C). Sera were shipped periodically in liquid nitrogen from Arua to the International Agency for Research on Cancer (IARC), Lyon, for long term storage in liquid nitrogen.

From February 1972 to September 1974, serum samples were obtained from about 42,000 children up to 8 yr old. The search for new cases of BL among those children was intensified from January 1973 by a team which visited regularly all health centres, dispensaries and hospitals in the survey area. All suspected cases were traced and examined clinically. If BL was still suspected, the following specimens were obtained before treatment: (1) a needle biopsy and touch smears for cytological evaluation; (2) a surgical biopsy fixed in formalin sent to the Department of Pathology of Makerere University, Uganda; (3) another biopsy sent to the IARC for coded evaluation by pathologists familiar with BL (G. O'Connor, National Cancer Institute and D. Wright, University of Southampton, with the help of N. Muñoz, IARC); (4) a piece of tumour, to be frozen, sent to H. zur Hausen's laboratory in Erlangen for detection of EBV/DNA<sup>21</sup>; (5) touch smears with fresh tumour tissue, air- and acetone-fixed, sent to IARC for detection of



**Fig. 1** Map of Africa showing the study area ---, County boundaries; ····, sub-county boundaries; - · - ·, parish boundaries; ○, BL cases.

**Table 2** Demographic, clinical and pathological data of the 14 BL patients who were bled before diagnosis

| Case no. | Sex | Diagnosis |            | Site of tumour       | Outcome         | Pathology              | EBV/DNA in tumour |
|----------|-----|-----------|------------|----------------------|-----------------|------------------------|-------------------|
|          |     | Age (yr)  | Date       |                      |                 |                        |                   |
| 202      | F   | 5         | May 1973   | R. mand./ovary       | Died Oct. 1973  | Confirmed              | ND                |
| 211      | M   | 5         | Sept. 1973 | Cervical/lymph nodes | Alive Dec. 1977 | Consistent             | ND                |
| 213      | F   | 3         | Sept. 1973 | R. mand./ovary       | Died Dec. 1973  | Consistent             | ND                |
| 216      | F   | 6         | Dec. 1973  | Both max.            | Died April 1974 | Consistent             | ND                |
| 221      | F   | 6         | Sept. 1974 | R. orbit             | Alive Dec. 1977 | Consistent             | 54 g.e.           |
| 222      | M   | 4         | Sept. 1974 | Both max./both mand. | Alive Dec. 1977 | Consistent             | 116 g.e.          |
| 226      | F   | 10        | Feb. 1975  | R. orbit/liver       | Died Feb. 1975  | Confirmed              | 49 g.e.           |
| 233      | M   | 5         | July 1975  | Orbits/liver         | Died Nov. 1975  | Consistent             | ND                |
| 234      | M   | 8         | July 1975  | R. max./BM           | Died Dec. 1975  | Consistent             | 30 g.e.           |
| 235      | M   | 10        | July 1975  | L. mand.             | Alive Dec. 1977 | Confirmed              | 38 g.e.           |
| 242      | M   | 12        | Sept. 1975 | L. mand.             | Alive Dec. 1977 | Consistent             | ND                |
| 247      | M   | 5         | Nov. 1975  | Orbits/liver         | Died Mar. 1976  | Consistent             | Negative          |
| 260      | M   | 5         | Nov. 1976  | R. orbit/R palate    | Died Nov. 1977  | Unclass.*              | Negative          |
| 263      | M   | 8         | Nov. 1976  | L. max./L clavicle   | Alive Dec. 1977 | lymphoma<br>Consistent | 40 g.e.           |

Mand., mandible; Max., maxilla; BM, bone marrow; R, right; L, left; ND, not done because of insufficient tumour tissue; g.e., genome equivalents per tumour cell (determined by nucleic acid reassociation kinetics<sup>21</sup>).

\*The three pathologists described this tumour as retinoblastoma, metastatic neuroblastoma and unclassified lymphoma, respectively.

EBV nuclear antigen<sup>22</sup>; (6) frozen serum samples from the child and other members of the family, and (7) thick and thin blood films for malarial burden comparison with films taken from the patient before diagnosis.

Three types of control were used for comparison with the serum of each new BL patient: (1) serum from a neighbour of the same age and sex selected at random from the main survey, who was revisited and bled again together with his whole family; (2) four control sera selected from the serum bank at

IARC taken from children of the same age, sex and locality as the BL patient and bled in the main survey at about the same date, and (3) sera from a random sample of the surveyed population<sup>23</sup>.

### Antibodies to VCA

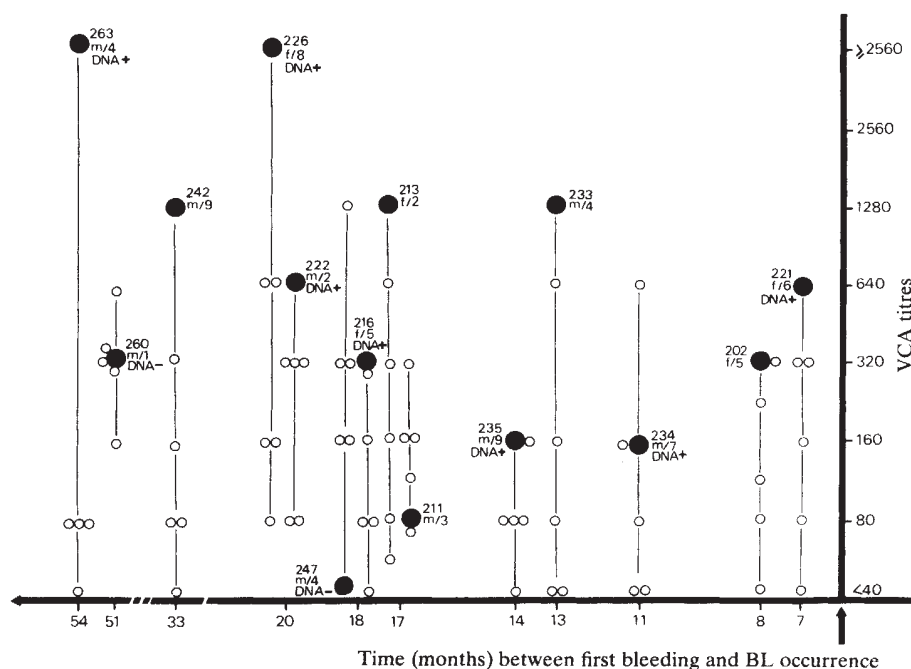
Between 1973 and November 1977, 31 children had been clinically diagnosed with BL in the study area (Table 1), but

| TITRE | EBV REACTIVITIES |         |                   |         |                   |         |                   |         |                   |         | HSV <sup>5</sup> |         | CMV <sup>6</sup> |         | MEASLES <sup>7</sup> |         |
|-------|------------------|---------|-------------------|---------|-------------------|---------|-------------------|---------|-------------------|---------|------------------|---------|------------------|---------|----------------------|---------|
|       | VCA <sup>1</sup> |         | EA-R <sup>2</sup> |         | EA-D <sup>2</sup> |         | EBNA <sup>3</sup> |         | CF/S <sup>4</sup> |         |                  |         |                  |         |                      |         |
|       | BL               | CONTROL | BL                | CONTROL | BL                | CONTROL | BL                | CONTROL | BL                | CONTROL | BL               | CONTROL | BL               | CONTROL | BL                   | CONTROL |
| >2560 | oo               |         |                   |         |                   |         |                   |         |                   |         |                  |         |                  |         |                      |         |
| 2560  |                  |         |                   |         |                   |         |                   | ..      |                   |         |                  |         |                  |         |                      |         |
| 1280  | ooo              | .       |                   |         |                   |         | ....              |         |                   |         |                  |         |                  |         |                      |         |
| 640   | oo               | iii     |                   |         | o 263             |         | ooo               | iii     |                   |         |                  |         |                  |         |                      |         |
| 320   | ooo              | iii     |                   |         |                   |         | oo                | iii     |                   | .       |                  |         |                  |         |                      |         |
| 160   | oo               | iii     |                   |         |                   |         | o                 | iii     | oo                | iii     |                  |         | oo               | .       |                      | .       |
| 80    | o 211            | iii     |                   |         |                   |         | ....              | oo      | iii               | o       | .                | o       | iii              | .       | ....                 |         |
| 40    | o 247            | iii     |                   |         |                   |         | oo                | iii     | oo                | iii     | o                | iii     | oo               | iii     | o                    | iii     |
| 20    |                  | .       |                   |         |                   |         | o                 | iii     | o                 | .       | oo               | iii     | ooo              | iii     | o                    | iii     |
| 10    |                  | ..      |                   |         |                   |         |                   | ..      | o                 | ..      | ooo              | iii     | oo               | ..      | oo                   | iii     |
| <10   |                  |         | ooo               | iii     | ooo               | iii     | .                 |         | ....              |         | oo               | iii     | o                | ..      | ooo                  | iii     |

**Fig. 2** Serological reactivities of 14 pre-BL and 69 matched controls. (1) Antibody titres to EBV structural antigens (VCA). Indirect immunofluorescence test<sup>24</sup>, IARC results. (2) Antibody titres to EBV early antigens, EA-R, restricted component; EA-D, diffuse component. W. H.'s results. (3) Antibodies to EBV nuclear antigen (EBNA), anti-complement immunofluorescence test<sup>22</sup>, IARC results. (4) Complement-fixing antibody titres to soluble EBV antigen<sup>27</sup>, IARC results. (5) Antibody titres to HSV structural antigens. Indirect immunofluorescence test<sup>28</sup>, P. F.'s results. (6) Antibody titres to CMV structural antigens. Indirect immunofluorescence as in ref. 25. (7) Complement-fixing antibodies to measles virus<sup>29</sup>.



**Fig. 3** VCA antibody titres in sera collected from BL cases before tumour manifestation and from controls. ●, BL cases (sex/age (yr)), EBV-DNA in tumour; ○, control.



only 14 originated from the bled population. Figure 1 shows the geographical location of these 14 cases. Fourteen BL cases is less than expected during a nearly 5-yr follow-up of the studied cohort. As Table 1 shows, there was a decline in incidence of BL during the study period. Of the 31 cases, only three were missed at the original bleeding, the rest were either in a region not yet covered by the field team, or were too old for inclusion.

Demographic, clinical and pathological data for these 14 patients are given in Table 2. Most patients were diagnosed soon (2–4 weeks) after the family had observed the first signs. The interval between initial serum collection and the onset of BL varied from 7 to 54 months (Fig. 3).

EBV serological testing<sup>22,24–27</sup> by immunofluorescence and complement fixation was carried out blind in the IARC laboratory (VCA, EA, EBNA and CF/s) in Lyon and in W.H.'s laboratory in Philadelphia (VCA, EA-R and EA-D, EBNA) for the 14 patients ('pre-BL' and 'post-BL' diagnosis), for the 13 randomly selected matched controls (pre- and post-; the serum for one control patient could not be found in the serum bank), for the 56 neighbour controls, and for family members. Detailed results are available on request to us. Figure 2 shows the antibody titres of pre-BL sera and matched controls.

Whereas no significant differences were observed for EA and EBNA reactivities between pre-BL sera and controls, higher titres of antibodies to VCA were seen in the pre-BL sera than in matched controls. The geometric mean titre (GMT) for both BL patients (425.5) and controls (125.8) was higher in IARC than in Philadelphia (BL patients, 176.7; controls, 52.7) but the ratio of the GMTs of patients and controls was about 3.4 to 1 in both places. Titres obtained at IARC have regularly been higher than those from Philadelphia because different cell lines were used as the source of antigen (Jijoye at IARC and EB3 in Philadelphia) and because of differences in the cut-off points taken in the immunofluorescent microscopy.

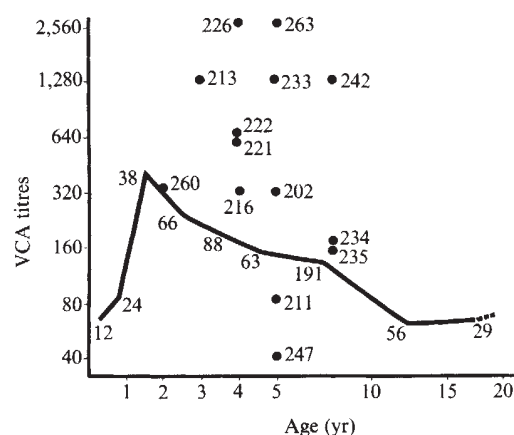
The differences in anti-VCA titres in pre-BL sera and in the sera of the 69 controls were statistically significant (IARC,  $t = 3.00$ ,  $P = 0.01$ ; Philadelphia,  $t = 2.65$ ,  $P = 0.02$ —two-sided tests). In the IARC results (Fig. 3), seven of the 14 pre-BL sera had a VCA titre higher than any of their controls, a further three had titres at least as high as any control, and the remaining four had VCA titres lower than their highest ranking controls. The probability (two-sided) of observing such a dis-

tribution by chance is 0.008 (non-parametric test), a value close to that obtained by the  $t$  test. The Philadelphia results gave almost identical values, as seven of the 14 cases had a higher VCA titre than corresponding controls.

In Fig. 4, the VCA titres found in children who were later to develop BL are compared with the GMTs of a random sample of the population in the survey area<sup>23</sup>. The titres of all but two of the patients are higher than the mean of those in the corresponding age group in the normal population. The risk of developing BL was estimated to be approximately 30 times higher for those with a VCA titre two dilutions or more above the normal population GMT standardised for age, sex and locality.

Detection of EBV genomes by nucleic acid hybridisation reassociation kinetics<sup>21</sup> was attempted in nine cases and was positive in seven (Table 2). In two cases, no EBV/DNA could be detected. Detection of the EBV-specific nuclear antigen (EBNA)<sup>22</sup> was attempted in eight biopsies and gave concordant results.

**Fig. 4** VCA antibody titres in sera collected from BL cases (●) before tumour manifestation compared with antibody titres in a random sample of the population of the study area (—). Numbers against solid line indicate number of sera tested at these ages in the random sample.



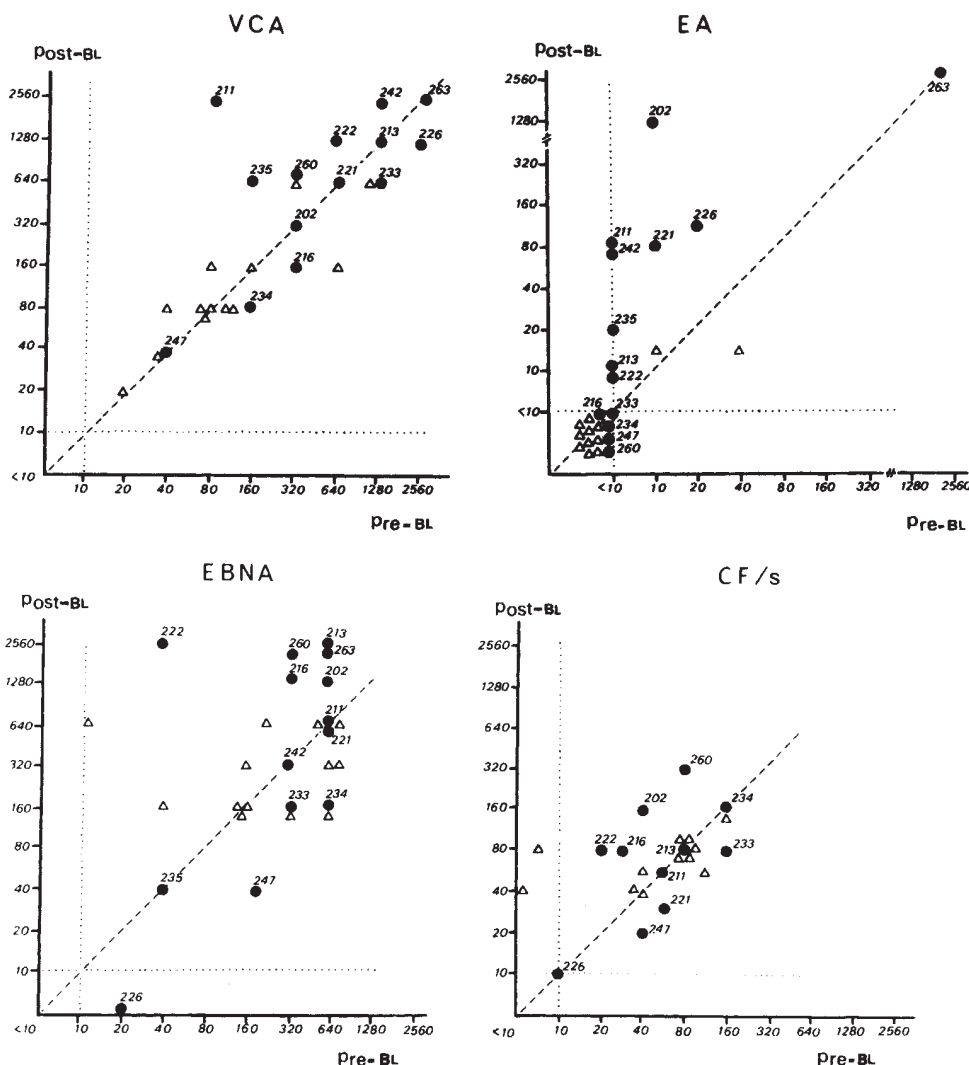


Fig. 5 EBV serological reactivities in cases (●) and controls (△) before and after clinical onset of BL.

## Serological activity and development of BL

Figure 5 compares EBV reactivities before and after the onset of disease. Antibody titres to VCA showed a remarkable stability, indicating that the high antibody levels previously associated with BL were present long before clinical onset. Of the other EBV activities, only EA antibodies developed, in eight of the 14 BL patients, subsequent to the initial bleeding. In seven of these, antibodies were directed against the EA-R component. One further case had very high EA-D antibody titres long before tumour development, which remained high after diagnosis (case 263). The EBV antibody titres of the randomly selected controls changed very little between initial bleeding and the clinical onset of BL in the matched patient. No differences were observed in sera from family members of cases compared to control families, taken at the main bleeding and after the disease onset of the case.

The pre-BL and post-BL sera as well as control sera were tested blind for immunofluorescent antibodies to herpes simplex virus (HSV) and cytomegalovirus (CMV) and complement-fixing antibodies to measles virus at the Center for Disease Control, Atlanta. Sera from BL patients before and after diagnosis showed no marked differences in antibody levels relative to those of controls (Fig. 2). These results are in contrast to those of Hilgers *et al.*<sup>30</sup>, who reported greater antibody titres to herpes viruses in sera from BL patients after clinical onset of disease than in sera from matched controls.

There was no marked difference between the number of malarial parasites in BL cases before diagnosis and in controls, but at diagnosis, patients had significantly fewer parasites than controls, possibly because they had taken anti-malarial drugs.

## Epidemiological evidence

Ten of the 14 patients had antibody titres to VCA as high as, or higher than, any corresponding controls long before tumour development. These results strongly support our third hypothesis that children with long and heavy exposure to EBV are at increased risk of developing BL. Elevated EBV antibody titres might indicate either that the virus is causally related to BL or merely that BL arises in persons with a longstanding defect in their cell-mediated immunity. If the latter were the case, elevated antibody titres to other herpes viruses, and to CMV in particular as it seems likely that this virus, like EBV, remains latent in B lymphocytes<sup>31,32</sup>. We therefore interpret our results as offering strong support for a causal relationship between EBV and BL.

Of the four BL patients who did not have elevated EBV antibody titres before disease onset, one (case 211) had a long history of tumorous cervical lymph nodes and exhibited antibodies to EA-D, characteristics atypical of BL. No tumour tissue was available from this patient to test for EBV markers. Case 260 was diagnosed as retinoblastoma, metastatic neuroblastoma or unclassified lymphoma by the pathologists and had no detectable EBV/DNA in the tumour tissue. The remaining two cases (234 and 247) had typical features of BL. Case 234



had detectable EBV/DNA in tumour tissue, while case 247 did not. Of the seven patients whose tumour tissue exhibited EBV markers, six had antibody titres to VCA before diagnosis as high or higher than their matched controls (Fig. 3).

The data suggest that the association of BL with predisease VCA titres is strongest in, and may be confined to, cases characterised by EBV/DNA in the tumour cells, and that among these cases high VCA titres are necessary for the disease. Thus it is possible that, in tropical regions, there are two types of BL with different aetiologies—one associated with EBV and the other not. The EBV-associated tumour would have high VCA titres long before clinical onset of disease and have detectable EBV genomes and EBNA in the tumour cells. The other lymphomas would not have abnormal VCA titres before onset of disease and their tumours would lack EBV markers (viral genome or EBNA). The latter would seem to constitute most childhood lymphomas in temperate climates<sup>13</sup>, while they are the exception in tropical areas, and their aetiology is unknown.

The lack of increase in anti-EBV activities other than VCA (EA, EBNA and CF/s) in BL patients before diagnosis might hinder interpretation of the higher VCA titres as evidence that EBV has a causal role in BL. But the various EBV antibodies do not develop simultaneously nor do they persist for equal periods after primary infection<sup>33</sup>. Antibodies to VCA appear soon after primary infection, first as IgM, then as IgG and the VCA titres tend to remain stable with only a moderate tendency to decline. EA antibodies also develop soon after primary infection, but decline rapidly and disappear within months. They would reappear in cases of reactivation of the latent infection, or possibly on reinfection (if this occurs). Antibodies to EBNA usually develop long after primary infection (a month to a year). Thus high VCA antibody titres may reflect the severity of the original primary infection, relating either to the infective dose or to the lack of proper host control due, for example, to an early age at primary infection or to a simultaneous severe attack of malaria. VCA antibody titres might then represent a marker for the remaining pool of EBV-carrying cells in a latent stage long after primary infection by the virus. The effect of oncogenic viruses in animal systems is enhanced when the virus is administered to newborn animals<sup>34</sup> and very early infection with the EBV has been suggested to initiate the induction of BL<sup>35</sup>. Our results are compatible with such a possibility. That pre-BL sera (with one exception) did not exhibit EA antibodies indicates that the long standing EBV infection was not chronically active.

## Need for other factors

It has been suggested<sup>36-38</sup> that BL arises as a result of immunological disorders in children exposed since early infancy to heavy malarial infection. The elevation of EBV/VCA antibody titres might accordingly be considered a consequence of failure to control the viral infection. High VCA titres in patients with Hodgkin's disease are believed to reflect impaired cell-mediated immunity<sup>39</sup>. But if this were the case for BL candidates, high titres of antibodies to CMV and HSV could have been expected, and these were not found either before or after diagnosis. The high antibody levels to VCA seems to be a specific phenomenon.

The time-space clustering and the seasonal variation (refs 18 and 40 and B. Lachet, N. E. D. and G. de T. in preparation) observed for BL imply a short interval (less than 18 months) between a final triggering event and the clinical onset of BL. Infection with EBV occurs too early to be the triggering factor and cannot be responsible for the geographical distribution of BL, but may be responsible for initiating the malignant process while holoendemic malaria or some other environmental factor(s) would promote the clinical manifestation of BL. A causal relationship between malaria and BL might be demonstrated by means of anti-malarial treatment and IARC has begun such a project in the North Mara district of Tanzania,

where chloroquine tablets are distributed twice a month to children aged 0-10 yr (ref. 42).

In conclusion, our results, taken in conjunction with the established *in vitro* transforming activity<sup>2,3</sup> of the virus and the demonstration of its oncogenicity in new world primates<sup>4,5</sup>, offer strong support for a causal association between EBV infection and the development of BL. However, as even in the West Nile district of Uganda less than one child in a thousand develops BL, other cofactors are required. It thus seems that the potential oncogenicity of EBV is realised only in exceptional circumstances. Final proof of causality would be the prevention of BL by intervention against EBV. The development and testing of a suitable vaccine<sup>43</sup> could be one means of achieving this, but considerable logistic and ethical problems would have to be surmounted<sup>44,45</sup>.

The Government of Uganda kindly permitted this study. For practical help and advice we thank R. Depue (NCI), G. Henle (Philadelphia), J. Higginson (IARC), G. Kafuko (East African Virus Research Institute), G. Klein (Karolinska Institute), A. Linsell (IARC), R. Morrow (Harvard School of Public Health), C. Olweny (Uganda Cancer Institute), R. Owor (Makerere University), M. Pike (University of Southern California) and M. Schneidermann (NCI). We thank the medical officers, other government officials, chiefs, elders and people of the West Nile district of Uganda for help and cooperation. We also thank the field teams for their contribution, and Colette Bonnardel, Bernadette Charnay, Marie-Claude Favre-Beaut and Marie-France Lavoue for data processing and serology testing. This study was supported by contract no. NO1 CP 43296 between the NCI and the IARC, and by grant (no. Ha 449/12).

Received 29 March; accepted 30 June 1978.

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# DNA sequence for a low-level promoter of the *lac* repressor gene and an 'up' promoter mutation

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*The promoter for a weakly expressed constitutive gene, the lactose repressor gene (*lacI*), has been sequenced, along with an 'up' promoter mutation *I*<sup>q</sup>. The 10-fold enhancement in *I* expression found in *I*<sup>q</sup> is the result of a single base change at position -35. To facilitate the sequencing, the *lacI* gene was cloned in a small plasmid.*

ALTHOUGH a promoter is functionally defined as the region of the DNA that precedes a gene and signals RNA polymerase to begin transcription, there is as yet no concise and universal definition of a promoter at the nucleotide level. Although many promoter DNA sequences are known, there is little homology between them. In the absence of data correlating each sequence to a given level of transcription, few hard conclusions about how promoter structure controls gene expression are possible.

The wild-type *I* gene, coding for the lactose repressor, has an unusually weak promoter that works constitutively. Genetic studies have generated a number of 'up' mutations in the *I* gene promoter, each resulting in an elevated level of *lac* repressor protein<sup>1-3</sup>. In correlating the exact nucleotide changes of these mutant promoters with the *in vivo* levels of repressor made by each, some critical determinants of promoter structure should become apparent. As the first step, the *I* gene DNA was made accessible by constructing a plasmid and the wild-type promoter sequence, together with the sequence of the 'up' promoter mutation, *I*<sup>q</sup>, were determined.

## *I* fragment

A transducing phage,  $\lambda$ h80dlac (ref. 4), which carries the *lac* operon including the entire *I* gene, was the source of *I* DNA. The *I* gene was cloned in the plasmid vector pMB9 (ref. 5).

In order to purify an *I* gene-containing fragment from the rest of a restriction enzyme digest of the  $\lambda$ h80dlac DNA, I took advantage of the proximity of the *lac* operator, which lies 80 base pairs from the right-hand end of the *I* gene (Fig. 1); the *lac* repressor will specifically bind all operator-containing DNA fragments to a nitrocellulose filter<sup>6</sup>.

Filter binding a complete *Hind*II + III digest of the purified  $\lambda$ h80dlac DNA with *lac* repressor yields a fragment 789 nucleotides long (Fig. 2a, b), which has been sequenced in this laboratory (A. Maxam, W. Gilbert, N. Chapman, G. Copenhaver, H. Donis-Keller, W. Herr, N. Rosenthal, unpublished). It includes the last quarter of the *I* gene and the beginning of the *Z* gene, with the entire *lac* control region in between (Fig.

1). A partial digest of the phage DNA was then made to find both the operator and the rest of the *I* gene on a single fragment. Figure 2c shows a family of partial digestion products, obtained by reducing the amount of enzyme and the time of reaction. The size of the first five partial fragments was estimated by comparison with the *Hpa*II fragments of  $\Phi$ X174 (ref. 7). ( $\Phi$ X DNA was a gift of J. Sims; *Hpa*II was provided by D. McConnell.) The partial fragments were designated  $\alpha$ - $\epsilon$  in ascending order of size, which ranged from 1,350 to 3,000 base pairs.

A given partial fragment would comprise the 789 fragment plus further DNA from the right side (*Z* gene), left side (*I* gene), or both. To determine the origin of each of the partial fragments, the pattern of filter-bound fragments from a partial *Hind*II + III digest of  $\lambda$ plac5 DNA was compared with that of  $\lambda$ h80dlac.  $\lambda$ plac5 (ref. 8) differs from  $\lambda$ h80dlac in that most of the *I* gene is deleted.

Thus partial fragments that appear identical in the two digests must be from the *Z* side only, while fragments from the *I* side of the  $\lambda$ h80dlac digest will be edited out of the  $\lambda$ plac5 pattern. As shown in Fig. 2d, the  $\alpha$  and  $\epsilon$  fragments appear in both the  $\lambda$ h80dlac and the  $\lambda$ plac5 digests; therefore they must come from the *Z* side. On the other hand,  $\beta$ ,  $\gamma$  and  $\delta$  are absent from  $\lambda$ plac5 and must contain *I* material. This information together with the sizes of the partial fragments yields a unique assignment of position and size to all the *Hin* fragments in the region, as shown in Fig. 3.

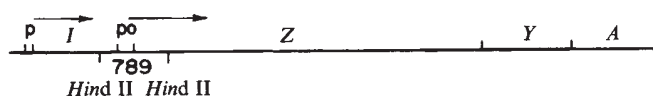
As the figure indicates, the next *Hin* fragment left of the 789 is 935 base pairs long. Since the *lac* repressor contains 360 amino acids (ref. 9 and amendment by P. Farabaugh in accompanying article<sup>10</sup>) as well as a 28 base pair untranslated leader region<sup>11</sup>, this 935 long fragment is big enough to contain the rest of the *I* gene and some 50 base pairs of the promoter.

## Cloning

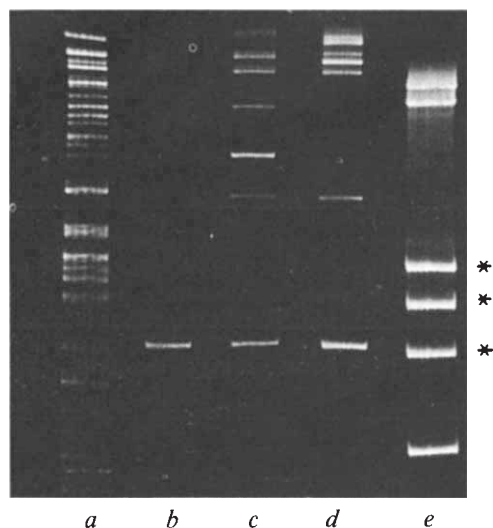
The  $\delta$  partial fragment is ideal for cloning. This fragment includes three *Hin* pieces: the 789 portion contains the operator, so that the whole fragment can be purified by filter binding; the 935 piece contains the bulk of the *I* gene and its promoter (on the assumption that the sequence out to -50 contains the promoter); and the 1,100 segment contains further sequences preceding the *I* gene. As determined by sequencing, the  $\delta$  fragment has half of a *Hind*II cutting site (GTYRAC)<sup>12</sup> on its right (789) end, and was likely also to have a *Hind*II site at its left end, since *Hind*II cuts greatly outnumber the more specific *Hind*III cuts (AAGCTT)<sup>13</sup>.

As a cloning vehicle pMB9, a readily amplified<sup>14</sup> ColEI derivative with tetracycline resistance<sup>5</sup>, provides a convenient site for insertion of a *Hind*III fragment. The single *Hpa*I cutting site in pMB9 has the sequence GTTAAC (ref. 15), a subset of the *Hind*II cutting sequence. Both enzymes give blunt rather than staggered ends. Thus if pMB9 is cut with *Hpa*I, each *Hind*II end from the  $\delta$  fragment can be linked to a *Hpa*I end from pMB9 with T4 ligase, closing the plasmid and regenerating a *Hind*II cutting sequence at each site of ligation. The legend to Fig. 2e gives details of the ligation and transformation.

**Fig. 1** The *lac* operon, to scale. The *Hind*II cuts in *I* and *Z* that give rise to the 789 fragment are shown. The arrows indicate the starting point and direction of transcription for *I* and *Z*.







**Fig. 2** *a*, Complete digest of  $\lambda$ h80dlac DNA with *Hind*II + III. (*Hind*II + III was provided by J. Sims). DNA was purified from a double lysogen (RV ( $\lambda$ h80cI857s7,  $\lambda$ h80cI857s7dlacuUV5)  $\phi$ 80<sup>r</sup>)<sup>4</sup> as in ref. 32, leaving out the sonication step. Digests were made at 37 °C in 6.6 mM Tris pH 7.4, 6.6 mM MgCl<sub>2</sub>, 2 mM DTT (dithiothreitol), and 60 mM NaCl, and run on a 20 × 20 × 0.2 cm polyacrylamide gel in TBE buffer (50 mM Tris borate pH 8.3, 1 mM EDTA) until the xylene cyanol marker ran off the bottom of the gel. The gel was stained for 20 min in 0.5  $\mu$ g ml<sup>-1</sup> ethidium bromide and photographed under ultraviolet light. *b*, Filter bound fragment from a complete digest of  $\lambda$ h80dlac. Fifty micrograms of the above digest was mixed with 5  $\mu$ g *lac* repressor (gift of R. Ogata) and filtered through a quarter sector S&S nitro-cellulose filter as in ref. 6. DNA was eluted from the filter in 50  $\mu$ l sample buffer (1/20 TBE, 10% glycerol, 0.01% xylene cyanol and bromophenol blue) containing 2  $\mu$ l 0.1 M IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) and loaded directly on the gel. *c*, Filter-bound fragments of a partial digest of  $\lambda$ h80dlac, purified as in *b*. The  $\delta$  fragment is faint in this photograph. *d*, Filter bound fragments from a partial digest of  $\lambda$ plac5, purified as in *b*. *e*, *Hind*II + III digest of pMC1. Starred bands derive from *lac* DNA (sizes 789, 935, 1,100 base pairs). The other three bands are from pMB9 (sizes approximately 600, 2300, 2600 base pairs). pMC1 was made by incubating 0.2  $\mu$ g *Hpa* I cut pMB9 DNA (gift of F. Fuller) with 0.5–1  $\mu$ g  $\delta$  fragment (purified from gels as in ref. 18) and 1  $\mu$ l T4 ligase (provided by N. Maizels) in 3  $\mu$ l 3 × ligase buffer (60 mM Tris pH 7.4, 25 mM MgCl<sub>2</sub>, 30 mM DTT, 0.18 mM ATP) in a total volume of 9  $\mu$ l for 1 h at 15 °C. The reaction was diluted to 33  $\mu$ l with 13  $\mu$ l ddH<sub>2</sub>O, 8  $\mu$ l 3 × ligase buffer, and 3  $\mu$ l T4 ligase and incubated for a further 2 h at 15 °C. 0.5 ml 0.5 M NH<sub>4</sub>Ac and 20  $\mu$ g carrier tRNA were added and the mixture was ethanol precipitated as in ref. 18. Five millilitres of competent cells, strain E7074 ( $\Delta$ (*lacpro*)<sub>XIII</sub>supEthi/*F'*lacI3,pro<sup>+</sup>) (Beckwith) were transformed with the ligated DNA in 50  $\mu$ l 30 mM CaCl<sub>2</sub>, as described in ref. 33, grown for 1 h in rich broth, and plated on minimal agar<sup>34</sup> without casamino acids and containing 10  $\mu$ g ml<sup>-1</sup> tetracycline and 40  $\mu$ g ml<sup>-1</sup> XG. Plasmid DNA was purified by the method of Tanaka and Weisblum<sup>35</sup>.

Plasmids that had acquired the  $\delta$  fragment were selected by screening transformants for the presence of a functional *I* gene. Transformation was done in an *i*<sup>-</sup> host strain, and the cells were plated on minimal agar containing tetracycline and XG (5-bromo-4-chloro-3-indolyl- $\beta$ ,D-galactoside), a chromogenic, non-inducing indicator of *Z* gene activity. Colonies of *i*<sup>-</sup> cells are blue on XG because *Z* gene expression is constitutive in the absence of the *I* gene product. Colonies that have acquired the recombinant plasmid will be white on these plates, since repressor is now made from the *I* gene carried on the plasmid.

Several white colonies were selected from the background of blue colonies on XG plates. One colony, named pMC1, was purified, grown up in large volume, and used as a source of

plasmid DNA. Digestion of the plasmid DNA with *Hind*II + III gives a pattern in which the three fragments derived from pMB9 are distinct from those derived from the *lac* region (Fig. 2*e*).

The 935 fragment makes up over 10% of the pMC1 plasmid and is easily purified in large amounts on preparative gels. Such a procedure has enabled P. Farabaugh to determine the DNA sequence of the *I* structural gene using pMC1 (ref. 10).

A similar plasmid containing the *I* gene promoter mutation *I*<sup>a</sup>, which makes 10 times the wild-type level of *lac* repressor<sup>1</sup> was created by genetically crossing the *I*<sup>a</sup> mutation from an *F'*lacpro episome to pMC1. The *I*<sup>a</sup> mutation was present on the *F'*lacpro starting episome used in a study of spontaneous *I*<sup>-</sup> mutations by J. Miller and coworkers (in preparation). Two *I*<sup>-</sup> mutations from this collection, *S58* and *S74*, which are located very early in the gene by deletion mapping, were crossed from the *F'*lacpro episome where they originated, to the plasmid by genetic recombination<sup>16,17</sup>. The *I*<sup>a</sup> promoter was assumed to be transferred with the *I*<sup>-</sup> mutation because of the close linkage involved.

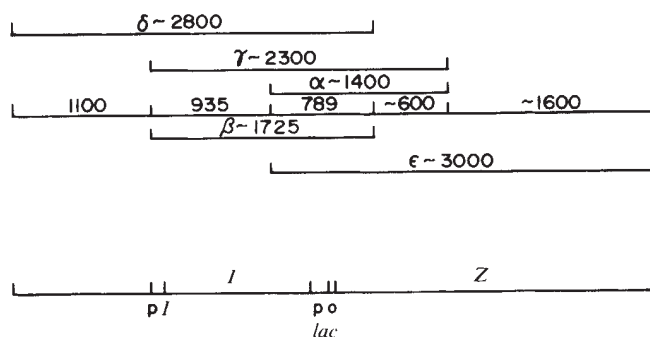
## Sequence

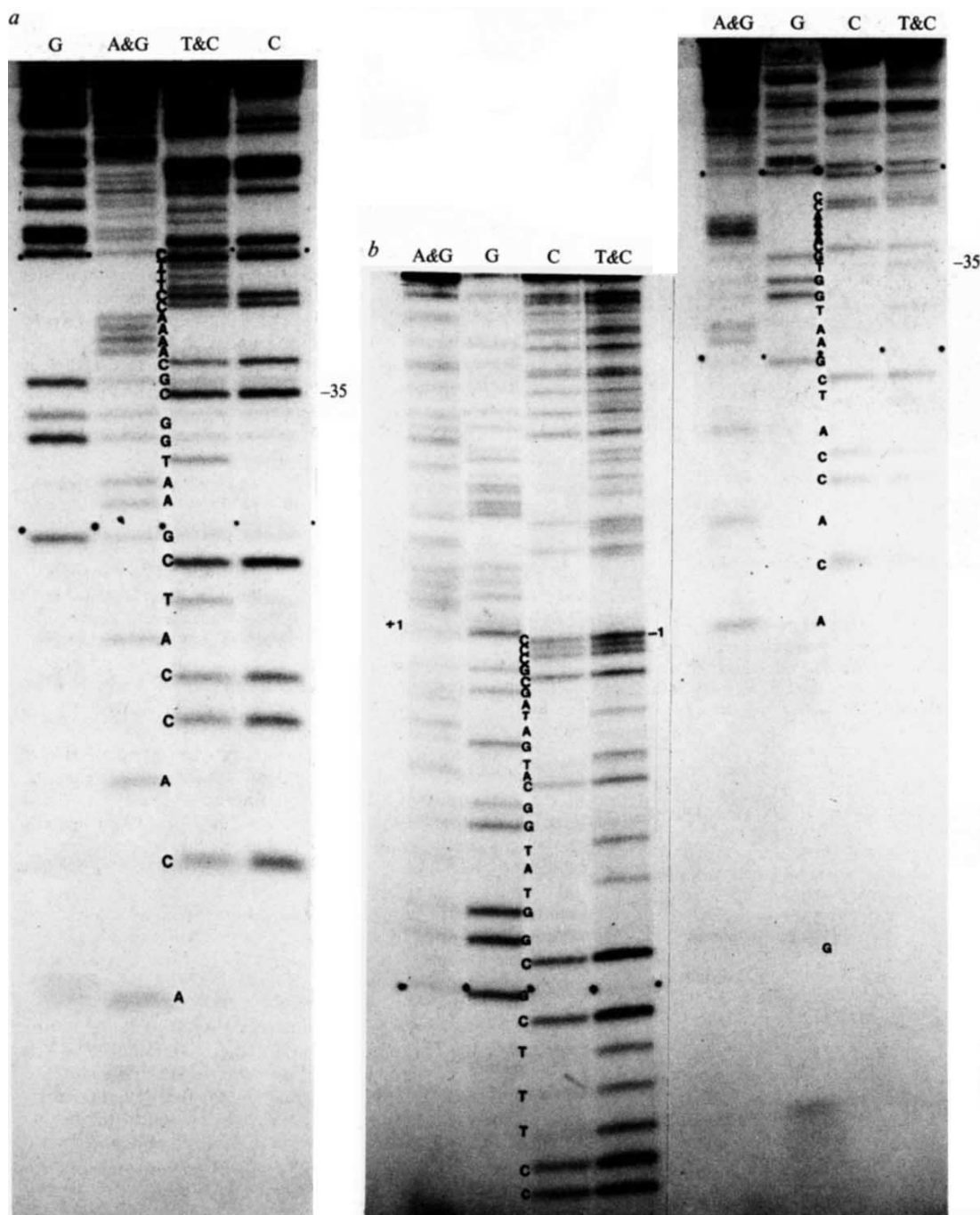
The promoter lies at one end of the 935 fragment. By labelling the 5' ends of the piece with <sup>32</sup>P and cutting it with each of several restriction enzymes, I obtained two end pieces of readily determined size for each enzyme. Without the previous knowledge of the DNA sequence of the structural gene, it was still possible to judge which end piece contained the promoter, using the information available from the amino acid sequence of *lac* repressor<sup>9</sup> and the first 168 bases of the *I* mRNA<sup>11</sup>. (A detailed restriction map is presented in ref. 10.)

The promoter was sequenced by the Maxam-Gilbert method<sup>18</sup>, which requires a fragment labelled on one end with <sup>32</sup>P. On different sequencing runs, different enzymes were used for making the secondary restriction cut in the 935 fragment labelled at both 5' ends with <sup>32</sup>P (Fig. 4). Strand separation of 50 and 67 base-pair long *Hpa* II pieces labelled at both 5' ends gave the sequence of the other strand. The 935 fragment contains 50 base pairs of DNA preceding the mRNA start. The sequence of the 28 base pair mRNA leader confirms the sequence previously reported (see ref. 11 for a discussion of this ribosome binding site). Figure 5*a* shows the DNA sequence of the wild-type promoter.

The length of the 935 fragment is altered in the plasmids containing the *I*<sup>a</sup> promoter linked to the *I*<sup>-</sup> mutations *S58* and *S74*. *S58* is an insertion of IS1 near the beginning of the structural gene<sup>16</sup>, thus the *I* gene fragment is lengthened by approximately 800 base pairs. *S74* is a deletion of the beginning of the structural gene, making the *I* fragment 75 base pairs shorter<sup>17</sup>. Neither mutation alters the promoter, however, so I sequenced the *I*<sup>a</sup> promoter from the appropriate *Hin* end

**Fig. 3** *Hind*II + III fragments in the *lac* region of  $\lambda$ h80dlac. Partials are labelled  $\alpha$ – $\epsilon$ . Approximate sizes are given in base pairs.





**Fig. 4** *a*, DNA sequencing gel of the wild-type *I* gene promoter from the *Hind*II cut. *b*, Sequencing gel of the *I*<sup>q</sup> mutation. The *I*<sup>q</sup> promoter changes C:G to T:A at position -35.

of the *I* fragment, as in the wild-type case. In both mutant plasmids, the promoter contains a single base change from C:G to T:A at position -35 (Fig. 5*b*).

### Sequence and function

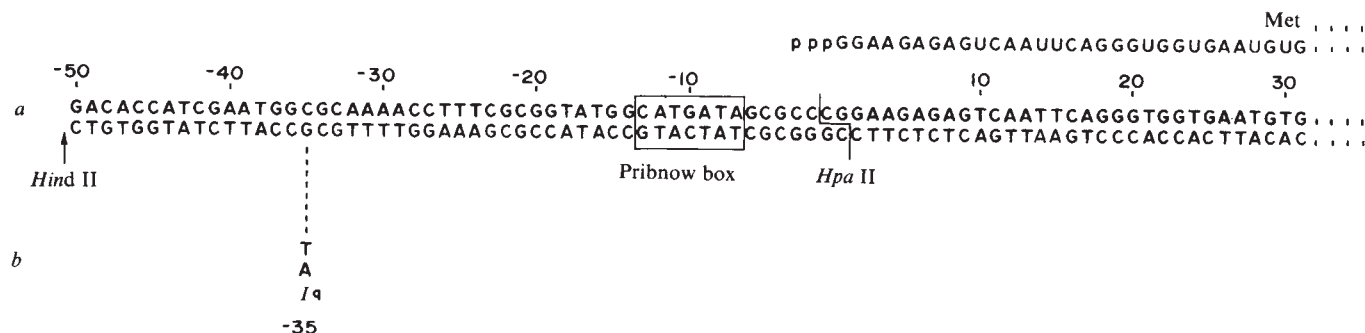
The wild-type *I* gene promoter is very weak. Transcription of the *I* gene, which is constitutive, occurs only once or twice per generation, producing about 10 repressor molecules per cell<sup>19</sup>. For comparison, transcription of the fully induced *Z* gene proceeds at more than 1,000 times this rate<sup>20</sup>. What features of the *I* promoter sequence render it such an unattractive substrate for RNA polymerase?

In comparison with other promoters, the sequence has the

homology region centred at -10, first noticed by Pribnow<sup>21</sup> and Schaller *et al.*<sup>22</sup>. It deviates from the prototype TATRATG sequence (Pribnow box) in two of the seven bases (CAT-GATA), a typical variation.

The composition of the spacer sequence between the Pribnow box and the mRNA start is unusual. If the Pribnow box were a site of DNA opening<sup>21</sup>, then it might be significant that this spacer sequence is composed entirely of G:C pairs in the *I* promoter, rather than the more easily opened A:T pairs. A similar sequence has been reported in the tRNA<sup>Tyr</sup> gene<sup>23</sup> and may contribute to the weak binding of polymerase to that promoter *in vitro*<sup>24</sup>. Overall, 28 of 50 base pairs in the *I* promoter are G:C, whereas most promoters have a majority of A:T base pairs. Sequence-specific contacts, however, rather than general melting behaviour, are doubtless of critical importance in promoter function<sup>25</sup>; yet the thermodynamic stability of crucial promoter segments, superimposed on an





**Fig. 5** a, DNA sequence of the wild-type *I* gene promoter and the 28 base pair leader that precedes the structural gene. b, The *I*<sup>q</sup> promoter changes C:G to T:A at position -35.

array of sequence-specific contacts, might be expected to affect promoter strength.

A region of homology in the vicinity of -35 has been noticed in many promoters<sup>25-27</sup>. Two mutations in the *lac* promoter, L305 and L241 (ref. 28), and two in  $\lambda$ ,  $\lambda$ <sub>P<sub>sex</sub></sub>1 (ref. 29) and  $\lambda$ <sub>PRM</sub>116 (ref. 30), have been sequenced and lie in the -35 areas, emphasising the importance of this region. All four mutations seriously impair their promoters. The sequence TGTTG is present in the -35 region of many promoters, and this homology region extends more weakly for another seven bases, ACAATTT being the best fit<sup>31</sup>. The *I* promoter, however, matches only two of the seven bases of the weaker homology region and, more importantly, deviates from the TGTTG sequence in the -35 region. When lined up to maximise homology with other promoters<sup>31</sup>, the *I* sequence in this region is TGGCG. The C in the fourth position is significant since this base is T in other promoters, being the most highly conserved base in the homology region. The 'up' promoter mutation *I*<sup>q</sup> changes the C to a T in this position, -35, bringing the *I* gene promoter into conformity with the other known sequences. The dramatic increase in efficiency between the wild-type and *I*<sup>q</sup> promoters clearly pinpoints a critical RNA polymerase contact at -35. This contact, which must be poor in the wild-type *I* promoter, is apparently stronger in *I*<sup>q</sup>, leading to an increased affinity between RNA polymerase and the promoter.

These sequences suggest that a poor contact at -35 and an abundance of G:C base pairs contribute to the relative indifference of RNA polymerase to the wild-type *I* promoter. The sequences of *I*<sup>sq</sup> and *I*<sup>q</sup> (refs 2 and 3), which are yet stronger 'up' promoter mutations, will point to additional defects in the wild-type promoter, and thereby further define the DNA sequence features necessary for active transcription.

I thank W. Gilbert for advice and discussions and J. Miller and M. Ptashne for comments on the manuscript. I was supported by an NSF predoctoral fellowship. This work was

supported by a NIGMS grant to W. Gilbert and was carried out within the framework of the guidelines established by the NIH for recombinant DNA.

Received 27 February; accepted 26 June, 1978.

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## Sequence of the *lacI* gene

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*The structural gene for the lac repressor of Escherichia coli, the lacI gene has been sequenced. This 1,080 base pair region of the E. coli chromosome codes for the lac repressor protein of 360 amino acids. The DNA sequence largely confirms but extends the previously reported protein sequence and allows a structural analysis of genetic phenomena.*

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USING the Maxam-Gilbert DNA sequencing technique<sup>1</sup>, the nucleotide sequence of the gene for *lac* repressor, *lacI* has been determined. A precise picture of the spontaneous and mutagen-induced spectra of *lacI*<sup>-</sup> mutations has been obtained by J. Miller and his colleagues<sup>2-4</sup> by the analysis of large numbers of deletions and point mutations of independent origin<sup>2-7</sup>. As in S. Benzer's study of the *rII* region of phage T4 (ref. 8), Miller and coworkers identify a variety of sites, called 'hotspots' by Benzer, which are unusually susceptible to mutagenesis<sup>2,4,5</sup>. Only the DNA sequence can explain how these 'hotspots' arise<sup>3,4</sup>.

The diagram illustrates the structure of the *lac* operon. The top section shows a detailed view of the DNA sequence with various regulatory sites labeled: D, PM, PH, A, H, TTM, AP, M, H, P, P, AMD, HA, HA. Arrows indicate the direction of transcription. The bottom section shows the overall map of the *lac* operon, including the *lacI* gene and the *lacZ* gene, with HindIII sites at 935 and 789 bp.

NH<sub>2</sub>-  
 MEFLYS<sup>10</sup>PROVAL<sup>20</sup>THRLEUTYRASPVALALAGLUTYRALAGLYVALSER<sup>30</sup>TYRGLN<sup>40</sup>THRVALSERARGVALVALASGLN<sup>50</sup>LALASERHIS<sup>60</sup>VALSERALALYSTHRARGGLULYSVALGLUALA  
 GTGAAACAGTAACTGATATCGCAGAGTATGCGGTGCTCTTATCAGACCGTTCCCGCGTGGTGAACAGGCCAGCCACGCTTCTGCGAAACCGCGGAAAAAGTGAAGCG  
 CACTTTGGTCATTGCAATATGCTACAGCGCTCATACGGCCACAGAGAATAGTCTGCGAAAGGGCGACCACTTTGGTCTGGTGGCAAGACGCTTTTGGCGCCCTTTTACCTTCG  
 HPAII MBII  
 ALAME<sup>50</sup>TALAGLULEUASNTYRILE<sup>60</sup>PROASNARGVALALAGLNGLNLEUALAGLYLYSGLN<sup>70</sup>SERLEULEU<sup>80</sup>ILEGLYVALALATHRSER<sup>90</sup>SERLEUALALEU<sup>100</sup>HISALAPRO<sup>110</sup>SERGLN<sup>120</sup>ILEVAL  
 GCGATGGCGGACTGAATTACATCCCAACCGCGTGGCACAACAAC<sup>130</sup>TGGCGGGCAACAGCTGTTGCTGATTGGCGTTGCCACCTCCAGTCTGGCCCTGCAACGGCGCGTGGCAAAATGTC  
 CGCTACCGCCTGACTTAATGTAAGGGTTGGCGACCGTGTGTTGACCGCCGTTTTCAGCAACGACTAACCGCAACGGTGGAGTGCAGACGGGACGTGGCGGGCAGCGTTTAAACAG  
 ALUI HAEIII  
 ALAALAILE<sup>130</sup>YSSERARGALASPGLNLEUGLYALASERVALVALSERMETVALGLUARGSERGLYVALGLUALACYSLYSALAALAV<sup>140</sup>ALHISASNLEULEUALAGLNARGVALSER  
 GCGGCGATTAAATCTCGCGCGATCAACTGGGTGCCAGCGTGGTGTCTGCTAGTGAACAAGCGCGCTGAGCGCTGTAAGCGGGGTGCACATCTCTCGCGCAACCGCTCAGT  
 CGCGCTAATTTAGAGCGCGGCTAGTTGACCCAGGTGGCACCACCACTGATACCATCTTGTTCGCCGCACTTCGGACATTCGCCGCACTGTTAGAGAGCGCGTGGCGAGTCA  
 TAQI MBII  
 GLYLEU<sup>150</sup>ILEILEASNTYRPROLEUASPGLN<sup>160</sup>ASPALALEALAV<sup>170</sup>GLUALAALACYS<sup>180</sup>THRASNVALPROALALEU<sup>190</sup>PHILEUASPVALSERASPGLN<sup>200</sup>THRPHILEASNSERILEILE  
 GGGGTGATCAATTAACATCGCTGGATGACCGAGTGCCTGCTGTGAAGCTGCCTGCCTCAATGTTCCGCGTATTCTTGTATGCTCTGACCGACACCCATCAACAGTATTATT  
 CGCGACTAGTAATGATAGCGCACTACTGGTCTACGTTCAACGACACCTTCGACGCGTGTATACAGGGCGCAAAAGTGTGTTGAGCTTACAGAGACTGGTCTGTGGGTAGTTGTCAATAAA  
 ALUI HPAII  
 PHSERHIS<sup>210</sup>GLUASPGLYTHRRARGLEUGLYVALGLUHI<sup>220</sup>SLEUVALALEU<sup>230</sup>GLYHISGLNGLNILEALALEULEUALAGLYPROLEUSERSERVALSERALAA<sup>240</sup>RGLEUARGLEUALAGLY  
 TTCTCCATGAAGACGGTACCGACTGGGCGTGGAGCATCTGTCGATTGGGTCAACGAAATCGCGCTGTTAGCGGCGCCATTAACTGCTGTCTCGCGCGCTGCTGGTCTGGCTGGC  
 AAGAGGTA<sup>250</sup>CTACTCTGCGATGCTGACCCGCACTCGTAGACGACGCTAACCACTGGTCTTTAGCGCGCAACATCGCCGGGAATTCAGAGACAGCGCGGACGACGACGACGCG  
 MBII HAEIII  
 TRPHISLYSTYNLEUTHRRARGASGLNILEGLNPROILEAL<sup>260</sup>GLUARGGLUGLYASPTRPSERALAMETSERGLYPHEGLNGLNTHRMETGLN<sup>270</sup>ILEUASGLNGLYILEVALPROTHR  
 TGGCATAAATATCTCACTCGCAATCAAAATTCAGCGGATAGCGGAACGGGAAGCGGACTGGAGTGCCATGTCGGTCTTCAACAAACCATGCAAAATGCTGAATGAGGGCATCGTCCCACT  
 ACCGATTTATAGAGTAGCGGTAGTTAGTTCGGCTATCGCCTTGCCTTCGCTGACCTCACGGTACAGGCGCAAAAGTGTGTTGGTACGTTACGACTTACTCCGCTAGCAAGGGTGA  
 HPAII  
 ALAME<sup>280</sup>TEUVALALAAASNAPGLNME<sup>290</sup>TALALEUGLYALAMETARGALALE<sup>300</sup>THRGLUSERGLYLEUARGVALGLYALASP<sup>310</sup>ILESERVALVALGLY<sup>320</sup>TYRASPPASPTHRGLUASP<sup>330</sup>SERSER  
 GCGATGCTGTTGCCAACGATCAGATGGCGCTGGGGCGAATGCGCGCCATACCGAGTCCGGGCTGGCGTGGTGGCGATATCTCGGTAGTGGGATACGAGTACCGAAGACACTCA  
 CGCTACGACCAACGGTGTCTAGTCTACCGCGACCCCGCTTACGCGCGGTAAATGCTCAGGCGCGACGGCAACCAACGCTATAGAGCCATCACCTATGCTGCTATGGCTTCTGTGAGT  
 HPAII ALUI  
 CYSTYRILE<sup>340</sup>PROPROLEUTHRRILELYSGLNASP<sup>350</sup>PHARGLEULEUGLYGLNTHRSERVALASPARGLEULEUGLNLEUSERGLNGLYGLN<sup>360</sup>ALAVALLYSGLYASNGLNLEULEUPRO  
 TGTATATCCCGCGCTGACCAACCATCAAAAGGATTTTCCGCTGCTGGGGCAACCCAGCGTGGACCGCTGCTGCAACTCTCTCAGGCGCAGCGGTGAAGGGCAATCACTGTGTTGCC  
 ACAATATAGGGCGGCACTGGTGGTGGTTGCTCTAAAGCGGACGACCCGCTTGGTGCACCTGGCGAAGCAGTGTGAGAGAGTCCGGTGCOSGCACTCCGCTTGTGTCACAAACGGG  
 MBII HINDII HAEIII ALUI  
 VALSERLEUALLYSARGLYSTHRRILEVALAPROASNTHRGLNTHRALASERPROARGALALEUALASP<sup>370</sup>SERLEU<sup>380</sup>METGLNLEUALAARGGLNVALSERARGLEUGLYSERGLYGLN<sup>390</sup>COOH  
 GTCTCACTGGTGAAGAAAAAACCACCTGGCGCCCAATACGCAAAACGCCCTCTCCCGCGCGGTGGCGGATTCATTAAATGCACTGGCAACGACAGTTTCCGCACTGGAAGCGCGGCACTGA  
 CAGAGTGACCACTTTCTTTTGTGGGACGGCGGGTTATGCGTTTGGCGGAGAGGGGCGCAACCGCTGAAGTAATAGCTGCAACCGCTGCTGCCAAGGGCTGACCTTTCCCGGCTCACT



In the preceding paper<sup>9</sup> Calos presents the sequence of the promoter for the *lacI* gene. I have extended the sequence through the *lacI* gene to the *lac* promoter/operator control region, which has been sequenced by Gilbert and Maxam (ref. 10 and personal communication) and Dickson *et al.*<sup>11</sup>. The repressor protein sequence of Beyreuther *et al.*<sup>12,13</sup>, a sequence for the initial portion of the mRNA reported by Steege<sup>14</sup>, and the DNA sequence of the terminal 223 base pairs of *lacI* constructed by A. Maxam with G. Copenhaver and W. Herr (personal communication) were known. The known protein sequence was used to position DNA sequences within the gene; the DNA sequence agrees with the protein sequence except for four emendations.

## Experimental design

The plasmid<sup>9</sup> which provided the DNA for this analysis consists of two DNA fragments covering the *lacI* region carried on the

pMB9 vector<sup>15</sup>. The *I* gene DNA was isolated by digesting the plasmid with *Hind*II+III (ref. 16) and separating the products on a 5% polyacrylamide (1:29 bisacrylamide) gel. The fragments containing *I* gene information are 935 and 789 base pairs long and correspond to the N and C-terminal portions of the protein respectively. Fragments resulting from cleavage with a second restriction endonuclease (either *Hae*III, *Hpa*II, *Alu*I or *Mbo*III (ref. 16)) were then labelled: treatment with bacterial alkaline phosphatase removes the 5' terminal phosphate, and T4 polynucleotide kinase catalyses the transfer of the radioactive phosphate of [ $\gamma$ -<sup>32</sup>P]ATP to the 5' termini of DNA to generate fragments with two 5' labels<sup>1</sup>. The Maxam-Gilbert technique requires singly-labelled DNA molecules; cleavage with a second restriction endonuclease or strand separation yielded two such molecules.

The sequencing technique<sup>1</sup> involves the modification of the nucleotide bases of these end-labelled fragments in a limited and random fashion using a purine-specific (dimethylsulphate, DMS) or a pyrimidine-specific (hydrazine) reagent. The

## Revised sequence for the *lac* repressor

THE elucidation of the DNA sequence of the *lacI* gene<sup>1</sup> has indicated the existence of an additional 13 amino acids not reported in the original protein sequence<sup>2</sup>. Recently a direct protein chemical verification of the suggested additional 11 amino acids in the repressor after position 147 and of 2 amino acids after residue 230 (formerly residue 219<sup>2</sup>) has been made. The strategy of the re-examination of the protein sequence included the isolation of [<sup>14</sup>C]carboxyamidomethylated cysteine-containing peptides which contain the sought-after amino acids and the automated Edman degradation<sup>3</sup> of these peptides. Thus, a quantitative detection and sequence determination was possible of all fragments including insoluble peptides since there was the suspicion that solubility problems and semi-quantitative manual sequencing methods<sup>2</sup> gave rise to the encountered discrepancies.

Repressor was cleaved at the two tryptophanyl residues 201 and 220 by BNPS-skatole<sup>4</sup> and one of the fragments thus

obtained was further digested with trypsin and subsequently with peptidyl-L-glutamate hydrolase (an endopeptidase cleaving specifically peptide bonds at glutamic acid residues)<sup>5</sup>. The results summarised in Fig. 1 are in complete agreement with the amino acid sequence predicted from the DNA sequence of the *lacI* gene<sup>1</sup>.

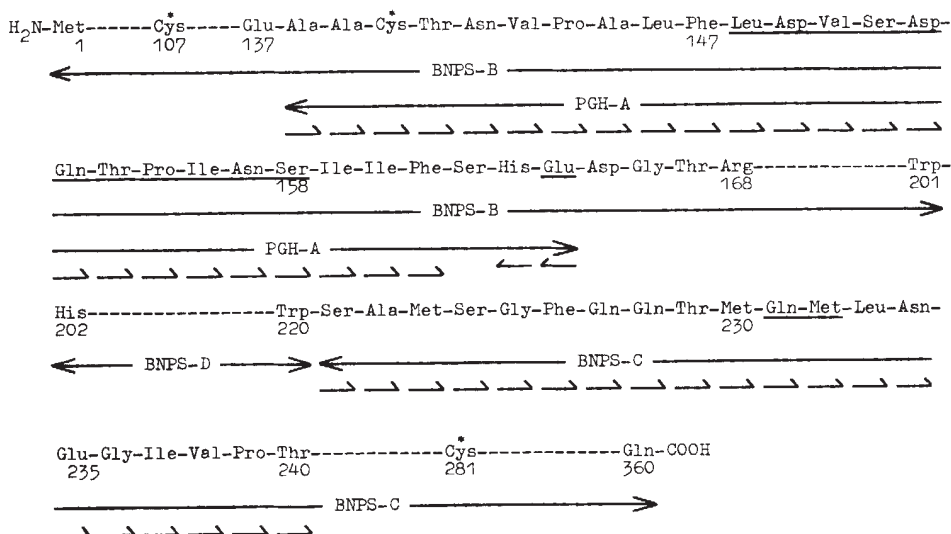
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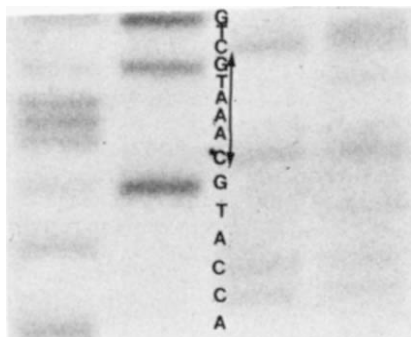
Received 27 February; accepted 26 June 1978

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**Fig. 1** Revised amino acid sequence of *lac* repressor of *E. coli*. Peptides are designated as BNPS when derived from cleavage with BNPS-skatole<sup>4</sup> or as PGH when derived from digestion with peptidyl-L-glutamate hydrolase<sup>5</sup>. The BNPS-fragments were separated on BioGel P150 in 6M guanidine hydrochloride. BNPS-fragment B was treated with trypsin, the largest tryptic peptide (residues 119-168) was isolated by chromatography on Sephadex G-50 in 0.05M NH<sub>4</sub>HCO<sub>3</sub> and cleaved with peptidyl-L-glutamate hydrolase. The PGH-peptides were isolated from the precipitate of the digest by chromatography on Sephadex G-25 in 2M acetic acid. A second radioactive PGH-peptide (PGH-B, residues 138-168) gave the same sequence 148-158 as shown for PGH-A. Residue positions determined by automated Edman degradation<sup>3</sup> (→) or by hydrolysis with carboxypeptidase C<sup>2</sup> (←) are indicated by arrows. The asterisk indicates [<sup>14</sup>C]carboxyamidomethylated cysteine. Automated sequence analyses were performed by using a Beckman updated model 890B Sequencer and a Quadrol (0.1 M) program<sup>2</sup>. Polybrene (Aldrich Chemical Co.) (0.7 mg per run) was used as carrier<sup>6</sup>. Identification of PTH-amino acids was as described<sup>2</sup>. The residues not reported in the original sequence<sup>2</sup> are underlined. Sequences 1-147, 159-230 (formerly residues 148-219) and 233-360 (formerly residues 220-347) with the exception of residue 164 are as reported previously<sup>2</sup>. The 360 residues of *lac* repressor



have a calculated molecular weight of 38,590.



**Fig. 3** Sequence of the six nucleotide pair 'insertion' corresponding to amino acids 231 and 232. A portion of the sequence of \**Hpa*108*Hpa* is shown.

modified bases are released and the chain broken at the site. Alteration of the conditions of either base modification or release enhances the reaction at one purine or pyrimidine relative to the other. Any one reaction contains a collection of fragments extending from the 5' end label to each of the several sites of specific strand scission, for example, each guanine in the chain. The products of the four reactions corresponding to the four bases are separated by electrophoresis through a 20% polyacrylamide (1:29 bisacrylamide), 7 M urea gel. Visualisation of the products by autoradiography results for each base in a band in the column or columns characteristic of that base starting at the 5' end and proceeding up the gel to the 3' end of the strand.

To identify the relevant fragments, I first constructed a restriction endonuclease map. The sequence of the 5' region of *I* mRNA<sup>14</sup> was valuable as it gave the position of the first *Hpa*II, *Hae*III, *Alu*I and *Mbo*II restriction sites in the region. Using the protein sequence of Beyreuther *et al.*<sup>12,13</sup> as an indication of the possible recognition sites, an unambiguous map was determined from the comparison of the patterns of cleavage of the *Hind*II fragments with one, or a pair of restriction endonucleases. The DNA sequence of a fragment was localised by translating it into the three reading frames and comparing the possible protein sequences with the repressor sequence; the DNA sequence of the gene confirmed the map. Figure 1 shows the detailed sequencing strategy.

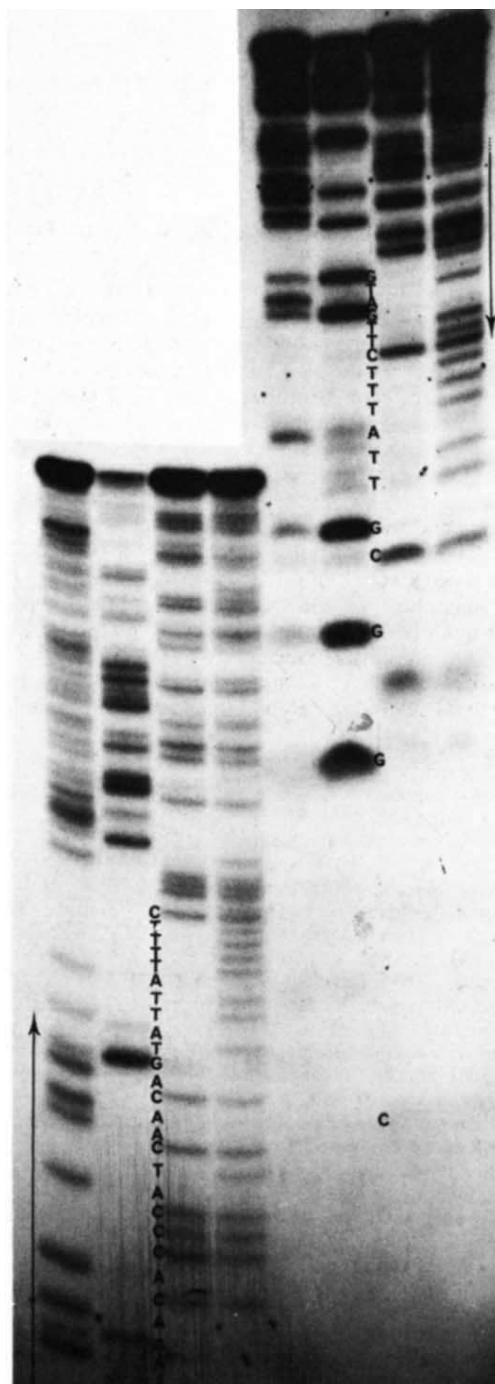
## DNA sequence

Figure 2 shows the sequence determined for the *lacI* gene and juxtaposes the deduced sequence of *lac* repressor. The DNA sequence runs from the initiation point of RNA synthesis, numbered 1 in Fig. 2 and which coincides with a *Hpa*II restriction endonucleolytic site, to the end of the coding region, the TGA termination codon, numbered 1,109 through 1,111. Figure 1 shows those regions of the gene which were sequenced directly; the other regions were assigned by base pairing rules. Calos (see the preceding paper) has sequenced the *lacI* promoter region, from the *Hind*II site at -50 to the beginning of the *lacI* coding region (+29). The sequences for the portion which we have both analysed, +1 to +29, agree. This sequence also agrees with that determined by Maxam, Copenhaver and Herr (personal communication) for the terminal 223 base pairs of *lacI*, from the second *Hind*II site to the terminator.

The only positions in the sequence which could be in error (marked with asterisks in Fig. 1) are five restriction endonuclease cleavage sites. Since I have not sequenced across these sites, the junction of the sequences on either side depends on two criteria—correspondence with the protein sequence of Beyreuther *et al.*<sup>12,13</sup>, and sizing on polyacrylamide gels of fragments which include the sites. Consideration of the available data indicates that if any regions were omitted, they would have to be extremely short.

The coding portion of the gene is 1,080 base pairs long, corresponding to a protein of 360 amino acids with a molecular weight of 38,350. As shown by Steege<sup>14</sup>, the coding region begins with a GTG initiation codon, which is preceded by a 28 base transcribed leader. Our sequences fully agree in the initial 211 bases of *I* mRNA. Her sequence contains several ambiguous regions which were correctly resolved by referring to peptide sequencing<sup>12,13,17</sup>.

The deduced sequence of *lac* repressor has several points of interest. Largely it confirms the sequence of Beyreuther *et al.*<sup>12,13</sup>, however there are discrepancies at four sites; in each



**Fig. 4** Sequence of \**Hpa*131*Hae* showing the region of the 33 nucleotide pair 'insertion' corresponding to amino acids 148 to 158.



|            |   | Second base                                 |                                 |                                              |                                                               |                  |            |
|------------|---|---------------------------------------------|---------------------------------|----------------------------------------------|---------------------------------------------------------------|------------------|------------|
| First base |   | U                                           | C                               | A                                            | G                                                             |                  | Third base |
|            | U | <i>Phe</i> { 3<br>1<br><i>Leu</i> { 5<br>5  | <i>Ser</i> { 7<br>6<br>3<br>5   | <i>Tyr</i> { 5<br>3<br><i>Term</i> { —<br>—  | <i>Cys</i> { 2<br>1<br><i>Term</i> { 1<br>1<br><i>Trp</i> { 2 | U<br>C<br>A<br>G |            |
|            | C | <i>Leu</i> { 2<br>3<br>1<br>25              | <i>Pro</i> { —<br>6<br>2<br>6   | <i>His</i> { 3<br>4<br><i>Gln</i> { 14<br>14 | <i>Arg</i> { 2<br>10<br>4<br>2                                | U<br>C<br>A<br>G |            |
|            | A | <i>Ile</i> { 10<br>7<br><i>Met</i> { 1<br>9 | <i>Thr</i> { 3<br>11<br>1<br>4  | <i>Asn</i> { 7<br>5<br><i>Lys</i> { 10<br>1  | <i>Ser</i> { 5<br>6<br><i>Arg</i> { 1<br>—                    | U<br>C<br>A<br>G |            |
|            | G | <i>Val</i> { 8<br>9<br>3<br>14+1            | <i>Ala</i> { 3<br>15<br>4<br>22 | <i>Asp</i> { 10<br>7<br><i>Glu</i> { 10<br>5 | <i>Gly</i> { 6<br>11<br>1<br>4                                | U<br>C<br>A<br>G |            |
|            |   | First base                                  |                                 |                                              |                                                               |                  |            |

**Fig. 5** Codon usage in *lacI*. The number of occurrences of each codon is indicated including the initiation (GUG) and termination codons (UGA). The amino acids whose spectra of codon usage show a probability of randomness of  $P < 1\%$  ( $\chi$ -squared analysis) are shown underlined. The usage of the four bases in the third position is U(76), C(103), A(61), and G(120); the random distribution, based on the amino acid composition, would be U(85.2), C(85.2), A(91.2), and G(98.2).

case the sequence coded by the DNA is shown in Fig. 2. In two positions, amino acids 164 and 215, I find the codon for glutamic acid (GAA) at a position assigned as glutamine (CAA). The other two discrepancies involve sites at which peptides were omitted from the protein sequence. A region of 33 nucleotide pairs (470–502; Fig. 3), and one of six nucleotide pairs (719–724; Fig. 4) appear in the middle of the coding region but do not correspond to any portion of the original sequence of Beyreuther *et al.*<sup>12,13</sup>

The two amino acid omissions around base 720 results from the loss of the fragments produced by cyanogen bromide cleavage at methionines 230 and 232. The 39-residue tryptic fragment from which it is derived includes six glutamine and glutamic acids, and five methionines; in this situation the loss of the Gln-Met peptide could easily have been obscured.

The second omission, the 11 amino acid peptide Leu-Asp-Val-Ser-Asp-Gln-Thr-Pro-Ile-Adn-Ser, is from a 50 residue tryptic peptide. The omission includes none of the five rarest amino acids in *lac* repressor; all residues lost occur at least twice elsewhere in the tryptic fragment. The omission of these residues would not necessarily be observed.

As the DNA sequence is unambiguous, the plasmid must contain these extra sequences. Beyreuther has recently confirmed this result by showing that the 11 amino acid peptide is present in proteolytic digests of *lac* repressor (see box).

The clear result of the direct analysis of protein, RNA and DNA sequences from *lacI* is that expression occurs in a linear fashion. There is a one-to-one correspondence between the RNA and the DNA sequences, and the DNA and the protein sequences. This colinearity in prokaryotes confirms results from other systems<sup>19–23</sup> and contrasts with the situation in eukaryotes where several examples of non-colinear expression have been reported<sup>24–27</sup>.

## Codon usage

The codon usage of *lacI* is non-random (Fig. 5). In several cases the gene shows a distinct preference for a subset of the possible codons. However, the most striking feature of the *I* spectrum is its lack of congruence with the other published examples of codon selection (MS2 (ref. 28) and  $\Phi$ X174 (ref. 29)). The two bacteriophages show a general preference,  $\Phi$ X strongly for XXU codons and MS2 less markedly for XXC codons; in *lacI* no such preference exists. The details of codon usage can be very different. In the case of leucine, 61% of the codons are CUG in the *lacI* gene. The predominant leucine codons of  $\Phi$ X and MS2 are CUU and CUC respectively, neither by as large a margin as CUG in *lacI*; both codons are minor species in *I*. A second strong bias is for the lysine codon AAA in *lacI* (10:1 over AAG); MS2 shows the opposite bias, though neither phage shows a strong preference. The only general trend in *I* is for XXG codons over XXA ( $P < 0.1\%$  by  $\chi$ -squared analysis). Again neither bacteriophage genome shows a similar pattern.

Both the general and detailed patterns of codon selection are significantly different. Although *lac* repressor is normally made in very small amounts, mutations at the *lacI* promoter raise the rate of synthesis<sup>30–32</sup>. No firm data exist on the question of relative translational efficiency in the three systems, but it seems that all are translated rapidly: the obvious differences in codon usage probably do not reflect a difference in efficiency.

This paper deals only with conclusions which can be drawn from the sequence alone. The implications of this sequence for the molecular origin of genetic phenomena will be explored elsewhere.

I thank Allan Maxam, John Majors, Lorraine Johnsrud, Michele Calos and Jeffrey H. Miller for discussions, and Walter Gilbert for guidance and support. This work was supported by a grant, GM09541, from the NIH to W. Gilbert, and was carried out within the framework of the guidelines established by the NIH for recombinant DNA.

Received 27 February; accepted 26 June; 1978.

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# Correlation of nonsense sites in the *lacI* gene with specific codons in the nucleotide sequence

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*Nonsense mutations derived from 90 different codons in the lacI gene of Escherichia coli have been correlated with the I gene nucleotide sequence. In over 80 cases the specific codon which generates the nonsense mutation can be identified. The sequence shows that 14–16 sites arise through tandem double base changes.*

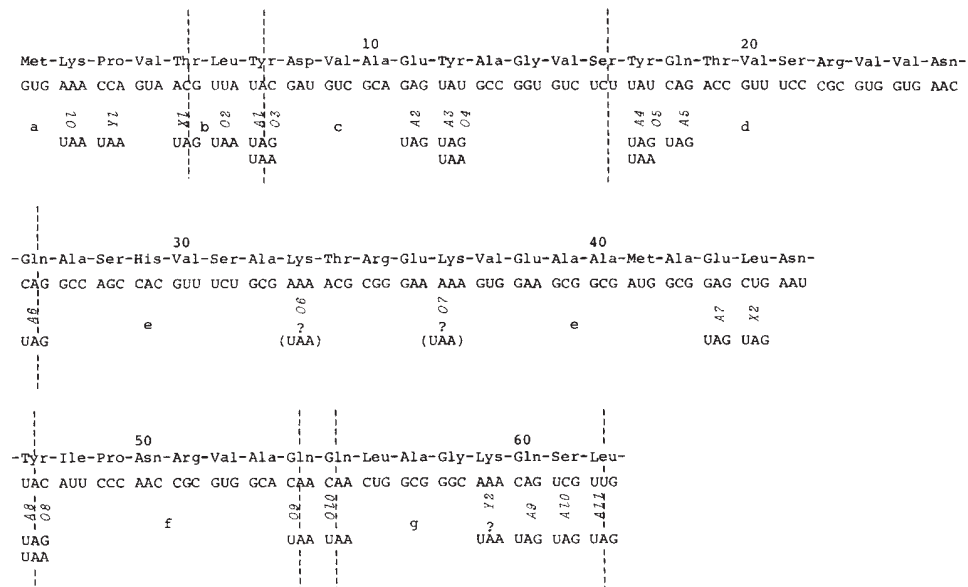
A LARGE collection of nonsense mutations at known positions in a gene makes possible a wide variety of experiments. Where the amino acid sequence has been determined, series of altered proteins carrying known sequence changes can be generated using characterised nonsense suppressors. The nonsense sites can also be used as markers to allow the correlation of the genetic and physical maps, allowing the localisation of missense mutations on the physical map simply by placing them in a specific deletion interval on the genetic map. In addition, one

could analyse the specificity of mutagens very precisely, as the exact base substitution involved in the generation of each nonsense site would be known.

More than 5,000 nonsense mutations in the *lacI* gene of *E. coli* have been characterised and divided into individual sites derived from 90 different codons<sup>1,2</sup>. By exploiting the specificity of 2-aminopurine and *mutT* and the knowledge of the *lac* repressor protein sequence, many of the nonsense sites were assigned to specific codons in the *lacI* mRNA<sup>1</sup>. This produced a partial correlation of the genetic and physical maps, which facilitated the assignment of additional nonsense sites to the relevant codons<sup>2</sup>. Now that the full nucleotide sequence of the *lacI* gene has been elucidated (preceding paper<sup>3</sup>), we can verify the specific codon predictions made in the case of each assignment. By pinpointing which codons can be converted to nonsense by a single base change, the sequence allows us to re-do most of the assignments simply and directly. Several correlations made from the protein sequence are ambiguous,

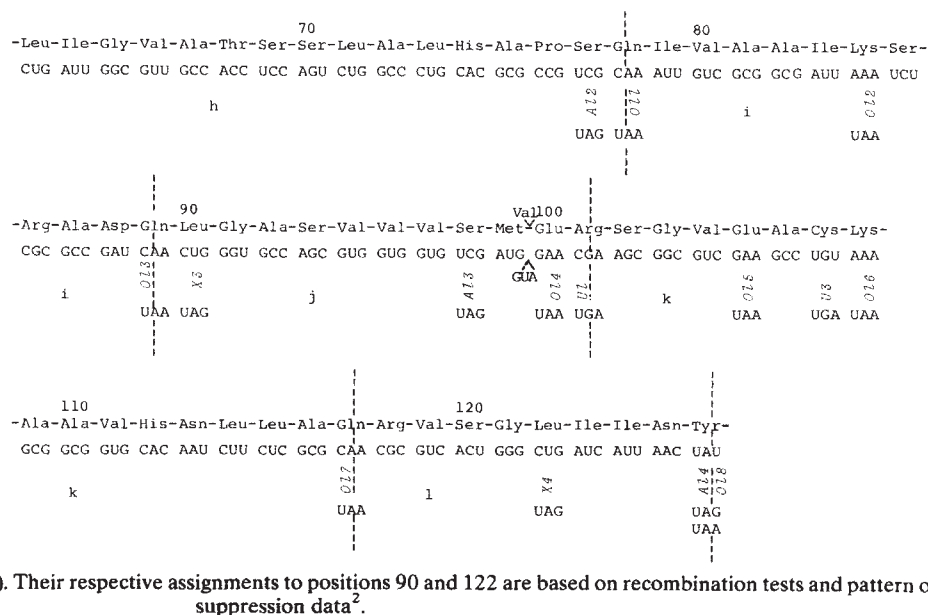
**Fig. 1** The gene-protein map from residues 1–62 is shown. The amino acid sequence in this and the subsequent diagrams is that reported by Beyreuther and coworkers<sup>4,19</sup>, as modified by Farabaugh<sup>3</sup>. The mRNA sequence was determined directly in the initial portion of the gene<sup>5</sup>, and deduced from the DNA sequence for the remainder<sup>3</sup>. Nonsense mutations are written directly below the codons to which they have been assigned, together with the respective allele numbers<sup>2</sup> (in italics). In most cases where the assignments are certain, the mutations are used as markers to fix the limits of intervals on the genetic and physical map. These boundaries are indicated by vertical dashed lines. Intervals are numbered with small letters. Thus, interval e extends from codon 26 to codon 47. The positions of missense mutations at codons 5 and 16 (refs 7, 20) have been determined by protein sequence studies.

These are also used as markers in this figure. Protein sequencing<sup>7,8,21</sup> has also verified the assignments of Y1, A1, A3, A4, A6, A9, A10 and A11 to positions 3, 7, 12, 17, 26, 60, 61 and 62, respectively. Within the intervals shown, most assignments can be achieved in a straightforward manner. Thus, position 18 is the only codon within interval d which can mutate to UAG by a single base change. The same is true for codon 11 in interval c, and for position 44 in interval e. Therefore, the correlation of these codons with the amber sites A5, A2, and A7, respectively, is direct, since these mutations arise from a single base substitution. By similar arguments O1 in interval a can be attributed to codon 2, and O2 in interval b to codon 6. Clearly, then, Y1, X1 and X2 must be derived by at least a double mutation. Protein sequencing<sup>21</sup> has shown that Y1 arises from a CC → UA change in codon 3. Mapping data and pattern of suppression evidence<sup>2</sup> place X1 at position 5 (indicating the AC → UA change) and X2 at codon 45 (a CU → UA change). In this and the following figures nonsense sites which seem to arise by a single base change are designated A and O, and those which are candidates for multiple base changes (usually involving tandem double base changes) are designated X and Y<sup>2</sup>. The exact assignments of O6 and O7 are not clear, as only two of the possible ochre sites in interval e have been found.





**Fig. 2** The portion of the gene-protein map from residues 63–126. All symbols are as described in the legend to Fig. 1. The only codon in intervals h and i which can be converted to amber (UAG) by a single base change is at position 77. This site can then be correlated with A12. Likewise, A13 can be assigned to position 97, the only single base change amber site in interval j. In a similar manner, O12 can be attributed to position 84, and the UGA site U3 to codon 107 in interval k. The three codons which can be converted to ochre (UAA) through a single base change in intervals j and k can be directly correlated with the three ordered ochre sites O14, O15, and O16. Sites X3 and X4 are not derived from single base substitutions, but rather arise from tandem double base changes in the same codon (see



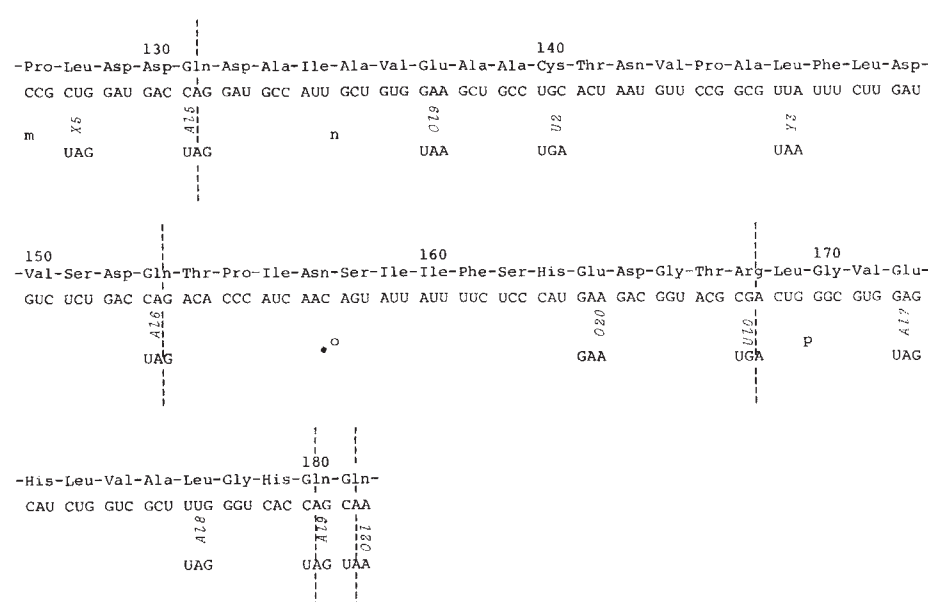
and some could not be made at all without the nucleotide sequence. We report here the completion of the correlation of close to 90 nonsense mutations with specific codons on the *I* mRNA. Experiments using this information are reported elsewhere.

## Correlation of nucleotide and protein sequence

Figures 1–6 show the *lac* repressor protein sequence and the corresponding mRNA sequence, either determined directly<sup>4,5</sup> or deduced from the DNA sequence<sup>6</sup>. The nonsense sites are lined up under the wild-type codon from which they are derived, with the appropriate allele numbers given in each case<sup>2</sup>. Here we consider each type of codon which was assigned in initial studies based on the amino acid sequence of the repressor<sup>1,2</sup>. The glutamine, tryptophan, tyrosine, and arginine codons will be discussed first. These have been assigned by experiments which relied heavily on the specificity of mutagens, and, in the case of several tyrosine codons, distinct patterns of suppression. A number of these assignments have

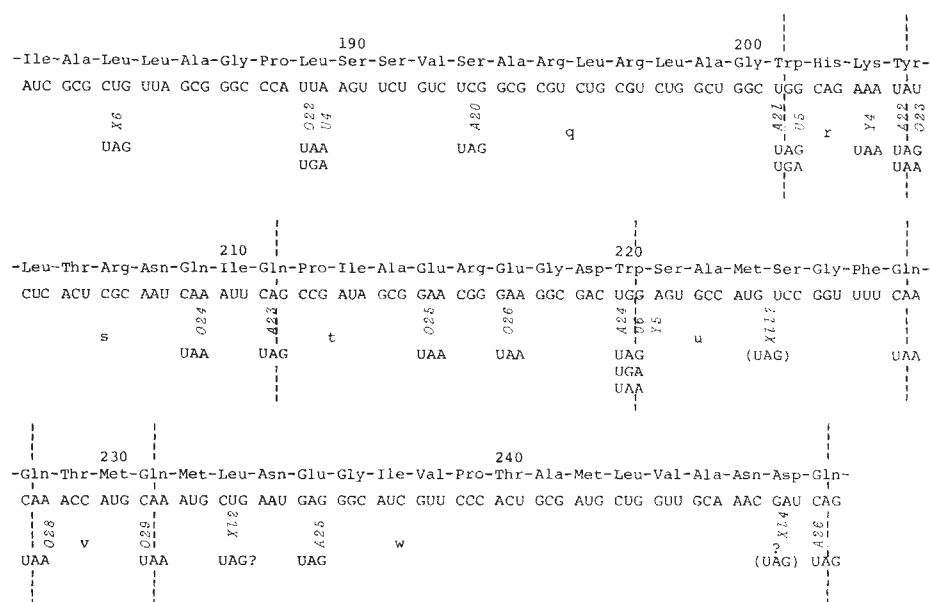
been verified by protein sequencing or other biochemical experiments<sup>7-9</sup>. The total set of codon predictions made by these assignments can be compared with the nucleotide sequence. The verification of these 36 assignments provides a group of known markers, which, together with an extensive deletion map<sup>10</sup>, divides the gene into more than 30 determined segments. Several other sequenced markers increase this total and additional deletions further subdivide these intervals<sup>10</sup>, allowing us to order mutations within the marked segments. Subsequently, nonsense mutations derived from serine, glutamic acid, leucine, lysine and cysteine codons will be considered. For these correlations we first compare the codon predictions made by the previous assignments<sup>2</sup> (based on the protein sequence) with those found in the nucleotide sequence. These studies were done with the handicap of having to take into account all of the possible codons given by the protein sequence which could generate nonsense mutations. Since the actual codons which can yield nonsense mutations are much more limited, we can show that the same correlations can be made in a straightforward manner using only the nucleotide sequence. This is described in the figure legends. In most cases

**Fig. 3** The portion of the gene-protein map extending from residues 127–181. (Amino acids 148 to 158 are as deduced in ref. 3.) Only two positions (172 and 177) can be converted to UAG by a single base change in interval p. The two amber sites in this region (A17 and A18) have been ordered and can be directly assigned to these respective positions. In interval o only the GAA at 164 can generate a UAA mutation in one step, leading to the assignment of O20 to this codon. There are two ordered ochre sites (O19 and Y3) which can be correlated with the only two available codons in interval n at positions 137 and 146. (It is not absolutely certain that Y3 is derived from a single base change, so its assignment to codon 146 is still tentative). This leaves position 140 as the only codon available for U2. The finding of an amber site inside interval m proves that more than a single base change was involved. The leucine-specific pa



ession suggests that this site (X5) is derived from the CUG codon at position 128, indicating a CU→UA tandem double base change.

**Fig. 4** Residues 182–248 of the gene-protein map, including the additional -Gln-Met- sequence at position 231 and 232, which was deduced from the DNA sequence<sup>3</sup>. In interval q, amber site A20 can be correlated with codon 193, the sole codon which can generate a UAG by a single step. Similarly A25 can be assigned to position 235, the ordered ochre mutations O25 and O26 to codons 215 and 217, and Y4 to position 203. (The ordering of Y4 in interval r is tentative.) The UAA/UGA pair O22/U4 must be derived from either position 185 or 189. The remaining mutations (X6, X11, X12, X14 and Y5) must be derived from at least double events. On the basis of pattern of suppression data<sup>2</sup>, X6 should be derived from a leucine codon, which is likely to be at 184, again implicating the CU→UA tandem double base change. X12 is probably derived from the same event at position 233. The origins of X11 and X14 are unknown at this point. (X14 cannot be precisely ordered with respect to A26.) Ochre site Y5 is derived from the UGG codon at position 220 (ref. 2) and arises from the GG→AA double base substitution.



only one assignment is possible when the specific codons are lined up with ordered amber, ochre, and UGA sites. This type of analysis can only be accomplished if the mutations are generated from single base changes. As is demonstrated later, some amber and ochre sites are derived by tandem double base substitutions. Virtually all of these are induced only by ultraviolet irradiation<sup>2,11</sup> and at a significantly lower rate than most single base substitutions, thus enabling us to distinguish them from single base changes in many cases. A number of these mutations can also be assigned to specific codons without directly sequencing each altered protein or gene.

### Glutamine codons (CAG, CAA) and tryptophan codons (UGG)

Amber and ochre mutations derived from glutamine codons were induced by 2-aminopurine<sup>1</sup>. On the basis of whether a UAG or UAA mutation was found, the set of assignments predicted the following codons: positions 18, 26, 60, 131, 153, 180, 211, 248, 291, 309, 311 and 317 = CAG. Positions 54, 55, 78, 89, 117, 181, 209, 227, 228, 231\*, 288 and 306 = CAA. Figure 1 shows that all 24 of these codon predictions are correct. Moreover, one CAA (marked with an asterisk, \*, at position 231) was predicted to occur in a region where the original protein sequence showed no glutamine residue<sup>1</sup>. As pointed out in the accompanying paper<sup>3</sup>, an extra AUGCAA (coding for Met-Gln-) is present in this part of the sequence. The amber assignments at positions 18, 26, 60 and 311 have been verified by independent biochemical experiments (refs 1, 7; and W. Gilbert and A. Maxam, unpublished results), as have those at the two tryptophan codons at positions 201 and 220 (ref. 9).

### Tyrosine codons (UAU, UAC)

Amber mutations were induced at five of the tyrosine codons by *mutT* (ref. 1). The resulting assignments predicted that the codons at positions 12, 17, 126, 204 and 282 are UAU. Spontaneous and 4-nitroquinoline-1-oxide induced mutations were attributed to the other three tyrosine codons at positions 7, 47 and 273 (ref. 2). This result, together with the inability of the respective amber mutations at these sites to be generated by the action of *mutT*, predicts that these three codons are UAC. The DNA sequence demonstrates that all eight tyrosine codon predictions are correct. Protein sequence studies have verified the position of the amber mutations assigned to codons 7, 12 and 17 (ref. 7).

### Arginine codons (CGX, AGG<sup>A</sup>)

UGA mutations induced by transition mutagens (2AP; EMS) can occur only at CGA (arginine) codons, and at UGG (tryptophan) codons. Experiments aimed at finding transition-derived UGA mutations in the repressor yield four different sites<sup>2</sup>, two of which corresponded to the characterised tryptophan codons. On the basis of the protein sequence<sup>3,4</sup>, the other two sites, U1 and U10, were assigned to arginine codons at positions 101 and 168. This predicts that CGA appears at these positions. In fact, out of 17 arginine codons in the first 350 positions, CGA appears only at codons 101 and 168. The DNA sequence verifies that these are the only positions in the respective marked regions which can generate UGA mutations through transitions.

### Serine codons (UCX, AGC<sup>U</sup>)

Only two of the six serine codons (UCG; UCA) can be converted to nonsense by a single base change. Amber mutations were attributed to serine codons at positions 61, 77, 93 or 97, 190 or 191 or 193, and 269 (ref. 2). The DNA sequence shows that the only UCG codons in the *I* gene are at positions 61, 77, 97, 193 and 269. This conforms nicely to the above predictions and eliminates the two ambiguities. The amber mutations at codons 77, 193 and 269 exhibit serine-specific patterns of suppression, which, together with their position relative to other markers was used in their assignments<sup>2</sup>. However, the same genetic data can be combined with the nucleotide sequence to effect their correlation with specific codons in a much simpler manner. To do this we rely on the markers discussed in the preceding sections. For instance, the nucleotide sequence shows that between the sequenced marker at position 62 and the assigned glutamine codon at position 89 only position 77 can generate an amber mutation by a single base change. There is strong genetic evidence that amber site A12, which maps in this interval (intervals h and i in Fig. 2), is derived from a single base substitution. It can therefore be attributed to the UCG codon at position 77, independent of the fact that it has a serine-specific pattern of suppression and maps extremely close to the ochre mutation assigned to position 78 (see legend to Fig. 2). This type of logic has been applied to the other serine codon assignments, and to all of the other nonsense assignments.

Two ochre sites have been correlated with serine codons, thus predicting a UCA at positions 279 or 280, and 322 (ref. 2). Figures 1–6 show that the only UCA codons in the first 340



amino acids are at positions 280 and 322. From the nucleotide sequence, these assignments follow directly from a comparison of the genetic data and the possible sites (Figs 4 and 5).

### Glutamic acid codons (GAG, GAA)

Amber sites have been assigned to glutamic acid codons at positions 11, 44, 172, 235, and 259 (ref. 2). The sequence shows that all five positions are indeed encoded by GAG and that these are the only GAG codons in the gene. In all five cases, the assignments agree with those made from considering the available codons in the sequence within each marked interval which can be converted to amber by a single base change and correlating them with the amber sites that are derived from a single base change. Ochre sites have been attributed to glutamic acid codons at positions 100, 105, 137, possibly 164, 215, 217 and 277 (ref. 2). This predicts that the codon at each of these positions is GAA, as is indeed the case. The figure legends describe the independent assignment of these sites.

### Leucine codons (CUX, UUA)

Amber mutations derived by a single base change have been correlated with leucine codons at positions 62 (which has been established by protein sequencing studies), 174 or 177, 304 or 305, and 318 or 319 (ref. 2). This predicts the position of UUG codons. In fact, the only four UUG codons in the sequence encoding the first 340 amino acids are at positions 62, 117, 304 and 319. We can therefore eliminate the ambiguity in the assignments of A18, A32 and A36 and correlate them with positions 177, 304 and 319, respectively. Ochre mutations have been attributed to leucine codons at positions 6, probably 146 or 148, and 189, based on comparing the genetics with the protein sequence<sup>2</sup>. The nucleotide sequence reveals four UUA codons in the portion of the gene specifying the first 340 amino acids. These occur at positions 6, 146, 185 and 189. The choice of position 189 instead of 185 for the correlation of O22 depends on mapping experiments which are reported elsewhere (ref. 2; see also Fig. 4).

### Lysine codons (AAG, AAA)

Only one amber site, Y8, has been attributed to a lysine codon<sup>2</sup>, thus predicting an AAG triplet at position 314. Figures 1-6 show that the only AAG codon in the *I* gene is at position 314. AAA codons are predicted to occur at positions 2, possibly 33 or 37, 59, 84, 108, 203, 290, and probably 325 or 327 (ref. 2). In fact, all of these positions are specified by AAA. The

validity of these assignments can be independently checked by considering the figures and figure legends.

### Cysteine codons (UGC, UGU)

UGA mutations can occur at cysteine codons by a single transversion. The mutagen 4-nitroquinoline-1-oxide, which induces transversions and transitions at G:C base pairs, stimulated UGA mutations (U2) which were correlated with the cysteine codon at position 140 (ref. 2). None of the other cysteine codons was affected by this mutagen, although UGA mutations were found after ultraviolet irradiation which were attributed to the cysteine codons at positions 107 and 281 (U3 and U8). These data suggest that the triplets at positions 107 and 281 are UGU, and the codon at 140 is UGC. The nucleotide sequence agrees with these predictions.

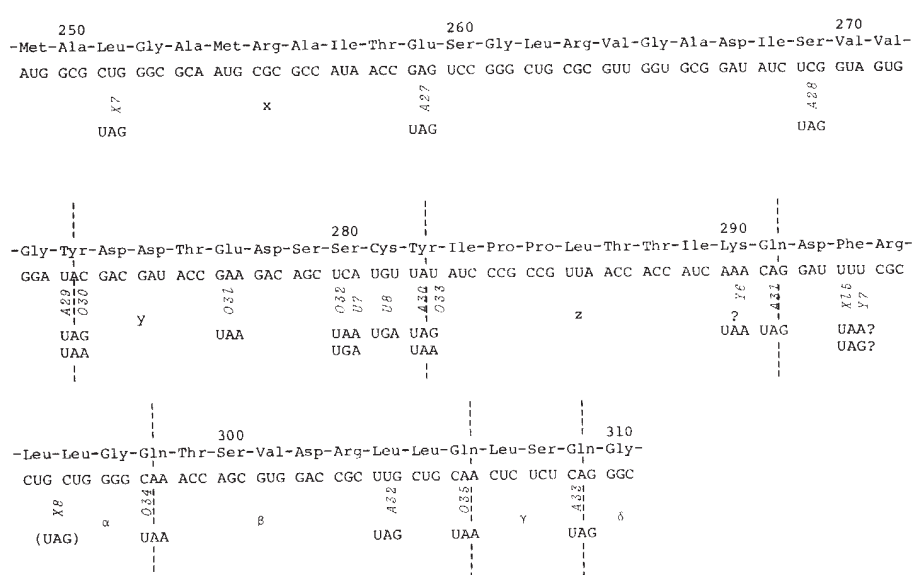
### Double mutations

Nonsense mutations apparently derived from tandem double base changes occur at 16 different codons. All of these are found after ultraviolet irradiation<sup>2</sup>, although at a lower rate than most single base changes. These are given X and Y designations (as opposed to A and O designations) in Figs 1-6. (Exceptions are X9, Y2, Y3, Y4, and Y8, which are probably due to a single base substitution.) In one case, Y1, the tandem double base substitution has been proved by direct protein sequencing (A. Schmitz, unpublished) and shown to involve a CC→UA change. Other changes which have been detected are: CU→UA (X2, X3, X4, X5, X6, X6, X8, X10, and X13); AC→UA (X1); CG→AA (Y5); UU→AA? (Y8); UU→AG? (X15); unknown multiple change (X11, X12, and X14).

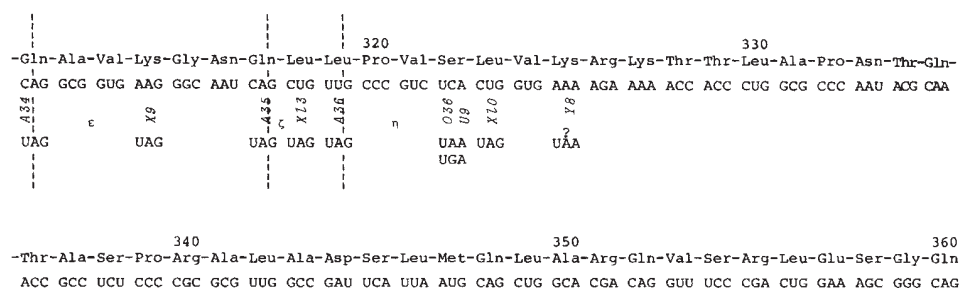
### Discussion

We have combined the results of genetic studies of the *lacI* gene with the full *I* gene DNA sequence and considered the 90 codons which have been converted to nonsense mutations. Most of the nonsense sites had been correlated with specific codons<sup>2</sup>. However, this work was based mainly on the protein sequence and left a number of ambiguities. We can verify the previous assignments by considering the nucleotide sequence, as the number of codons which can actually be converted to nonsense by a single base change is far fewer than the possible number predicted by the protein sequence. One measure of the accuracy of the previous assignments can be scored by checking each codon prediction against the sequence given in Figs 1-6. Thus, an amber mutation (UAG) attributed to the serine codon

**Fig. 5** Gene-protein map between residues 249 and 310. In interval  $\alpha$  only positions 259 and 269 can mutate to UAG by a single base change, allowing us to assign the ordered UAG mutations A27 and A28 to these respective codons. (This assignment of A28 to position 269 has been verified by DNA sequencing; P. J. Farabaugh, unpublished experiments.) By the same logic, O31 and O32 in interval  $\gamma$  can be assigned to positions 277 and 280, respectively, and A32 in interval  $\beta$  can be correlated with codon 304. Similarly, U8 must be derived from codon 281. If Y6 is derived from a single base change, it probably corresponds to position 290 based on mapping experiments<sup>2</sup>. On the other hand, X7, X15, Y7, and X8 must involve at least double base substitutions. The leucine-specific pattern of suppression of X7 and X8 suggests the CUG codons at 251 and 295 or 296 as the wild-type triplet. X15 and Y7 are at the same codon and could be generated by a different tandem double base change at the UUU codon at position 292, although this is tentative.



**Fig. 6** Gene-protein map from residues 311 to 360. Position 319 is used as a marker on the basis of DNA sequence results<sup>22</sup> which demonstrate that A36 involves a U→A change at this codon. In interval  $\epsilon$ , X9 can be correlated with codon 314, provided it is derived from a single base change. X13 is in interval  $\zeta$  and must originate from the CU→UA tandem double base change. In this region of the gene, no nonsense sites after position 348 can be detected in this system<sup>1</sup>. Both a UGA (U9) and a UAA (O36) mutation have been found at the same codon, enabling us to assign it to the UCA at position 322. This is the only codon between position 319 and 348 which can be converted to both UGA and UAA in one step. Amber site X10 maps very close to O36 and has a leucine-specific pattern of suppression. We have therefore assigned it to the CUG codon at position 323. There is no unambiguous correlation for the ochre site Y8, although the lysine codon at position 325 is most probable.



at position 77 predicts that UCG appears at this position (provided that the amber was generated by a single base substitution). From this type of analysis the tyrosine, glutamine, tryptophan and arginine codons (which were originally assigned by highly accurate methods) seem to be correctly correlated with the respective nonsense mutations. Moreover, a number of these sites have been verified by direct sequencing (refs 7-9; and W. Gilbert and A. Maxam, unpublished results). We therefore consider them as 'markers'. Together with several sequenced missense mutations, these markers have been used to divide the gene into 32 known intervals (Figs 1-6). From this point we can use the DNA sequence to assign most of the nonsense sites by simply lining up each ordered mutation with the codons which can be converted to amber, ochre or UGA. A comparison of the two methods demonstrates the accuracy of the assignments made in the previous study<sup>2</sup>.

The genetic studies also predicted the two additions in the protein sequence which were detected by the DNA sequence analysis<sup>3</sup>. An additional glutamine (CAA) was required to explain the ochre sites induced by transition mutagens near the tryptophan codon at position 220 (ref. 1). The preceding paper shows that an extra -CAAATG-sequence is present in this region of the gene<sup>3</sup>. In the portion of the gene between the glutamine codon at position 153 and the arginine codon at the original position 157, an ochre mutation derived from a single base change was detected<sup>2</sup>. The reported -Asp-Gly-Thr-sequence<sup>4</sup> between these two markers forbids this situation, as none of these residues is specified by a codon which can generate UAA by a single base substitution. Together with recombination data which suggest that the interval between these markers is larger than 13 nucleotides<sup>2,10</sup>, this finding is inconsistent with the original protein sequence. The presence of an additional 11 residues in this region of the gene has been deduced from the DNA sequence<sup>3</sup>, resulting in the arginine marker being assigned to position 168.

The genetic work which led to this analysis involved the characterisation of more than 5,500 independently derived nonsense mutations in the *lacI* gene<sup>2</sup>. Work with transition mutagens demonstrated that nonsense sites after position 335 could not be detected under the conditions used<sup>1</sup>, probably due to a partial activity of the resulting repressor fragments. (The  $I^O$  mutation<sup>12</sup> is used in the starting strain, causing a 10-fold overproduction of the repressor. We can use the DNA sequence to compare the number of nonsense sites found with the number of possible sites, and thus assess the degree of saturation of the map. In the region encoding the first 335 residues of the protein there are 37 codons which can be converted to UAG (amber) through a single base change. Nonsense mutations corresponding to each of these codons have been detected in the above collection. Out of 45 or 46 possible ochre sites, 41 have been found. There are several reasons for the failure to detect mutations corresponding to the codons at positions 185, 286, 327? and two of the four codons at positions 33, 36, 37 and 39. The leucine and lysine codons

(positions 185, 286, 33, 37 and 327) are converted to UAA through the A:T→T:A transversion, which is stimulated only by ultraviolet, among the mutagens used<sup>2,11</sup>. Therefore, any of these sites which happen to be poorly induced by this mutagen would not be represented. This could be overcome by either examining a larger collection of ultraviolet-induced ochre mutations, or by using a different mutagen which favours the A:T→T:A transversion. The failure to restore adequately the  $i^+$  phenotype by suppression could be responsible for some of these missing ochre mutations. Many of the leucine residues in the repressor cannot be substituted by other amino acids without the loss of significant repressor activity<sup>2</sup>. Because we do not have an ochre suppressor which inserts leucine, it is possible that ochre sites at positions 185 and 286 were indeed present in the collection of  $i^-$  mutations but not recovered as ochre mutations. This same explanation could also hold for each of the other sites, since glutamic acid codons are at positions 36 and 39 and we have no suppressor which inserts this amino acid. Also, the lysine inserting suppressor, Su5, is extremely inefficient<sup>13</sup>, and may not allow sufficient amounts of protein to be produced in a few cases. This may result in the failure to detect ochre mutations at positions 33 or 37; the negative complementation produced by reinitiation fragments in the region is particularly high<sup>2,7</sup> and this would tend to counteract the effects of low level suppression. Finally, position 327 may not yield a nonsense fragment which is completely  $i^-$  when overproduced, which would account for the apparent failure to find an ochre mutation corresponding to this codon.

Suppression of UGA sites involves tation of tryptophan at the nonsense site<sup>14</sup>. Therefore, we can only recover UGA mutations at positions in the protein which allow this amino acid exchange. In the portion of the gene encoding the first 335 residues, we have detected the UGA sites derived from both tryptophan codons, all 3 cysteine codons, both UCA serine codons, and both CGA arginine codons. Only in the case of UUA leucine codons have we failed to detect UGA mutations. In fact, out of five UUA codons, only that at position 189 has been detected as a UGA mutation. Even *mutT*, which specifically induces the A:T→C:G transversion<sup>15</sup>, fails to stimulate UGA mutations at these sites. It is probable, therefore, that tryptophan is partially or completely unacceptable at the corresponding position in the protein.

A number of sites seem to arise by tandem double base changes within the same codon. Out of 90 codons which yielded nonsense mutations, 16 fall into the above category. Of the mutagens used, only ultraviolet generated tandem double base changes as a significant percentage of the total induced mutations. Many of these double substitutions occur at CUG leucine codons, the CU→UA (or, on the DNA level, the C:G/T:A→T:A/A:T) change being involved. Tandem double base changes induced by ultraviolet have been described in a number of other systems (refs 16, 17, and J. Gralle, unpublished results). The relevance of these findings to the mechanism of ultraviolet mutagenesis is discussed elsewhere<sup>11</sup>.



Elsewhere we report the use of this system to explore some biological questions. By measuring the induction of specific nonsense sites, base substitution mutations can be assayed<sup>11</sup>. The relationship between the surrounding nucleotide sequence and the rate of mutation can then be examined with the hope of uncovering the molecular basis of genetic hotspots<sup>18</sup>. The nonsense collection has been used, together with different nonsense suppressors, to generate over 300 altered repressor proteins. The properties of these repressors are being examined, to increase our understanding of the relationship between repressor structure and function.

We thank Drs Walter Gilbert and Sydney Brenner for helpful discussions. This work was supported by a grant from the Swiss National Fund (3.179.77) to J.H.M. and by grant GM09541 from NIH to W. Gilbert.

Received 27 February; accepted 26 June, 1978

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# Molecular basis of base substitution hotspots in *Escherichia coli*

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*In the lacI gene of Escherichia coli spontaneous base substitution hotspots occur at 5-methylcytosine residues. The hotspots disappear when the respective cytosines are not methylated. We suggest that the hotspots may result from the spontaneous deamination of 5-methylcytosine to thymine, which is not excised by the enzyme DNA-uracil glycosidase.*

IN 1961 Benzer demonstrated that all sites on DNA are not equally mutable, and termed highly mutable sites 'hotspots'<sup>1</sup>. We have studied base substitution mutations in the *lacI* gene of *E. coli*. Because deletions, insertions, and small frameshifts constitute ~90% of the mutations arising spontaneously in the *I* gene<sup>2</sup>, we selected directly for base substitutions by focusing on nonsense mutations<sup>3</sup>. Over 80 of the characterised nonsense sites are correlated with specific codons<sup>4,5</sup>; thus in each of these cases the conversion to UAG or UAA occurs by a known base substitution. This system has been used to analyse the specificities of base substitutions detected spontaneously and after induction by various mutagens<sup>3</sup>.

Several striking hotspots appear in this collection of mutations, particularly among spontaneous base substitutions, and among those generated by 2-aminopurine (2-AP) and ultraviolet light. The elucidation of the full nucleotide sequence of the *lacI* gene<sup>6,7</sup> now allows the examination of the DNA sequence surrounding each site of mutation. It is clear that 5-methylcytosine residues are associated with high mutability for the G:C→A:T change, and we describe here several experiments which demonstrate the direct involvement of 5-methylcytosine in causing base substitution hotspots.

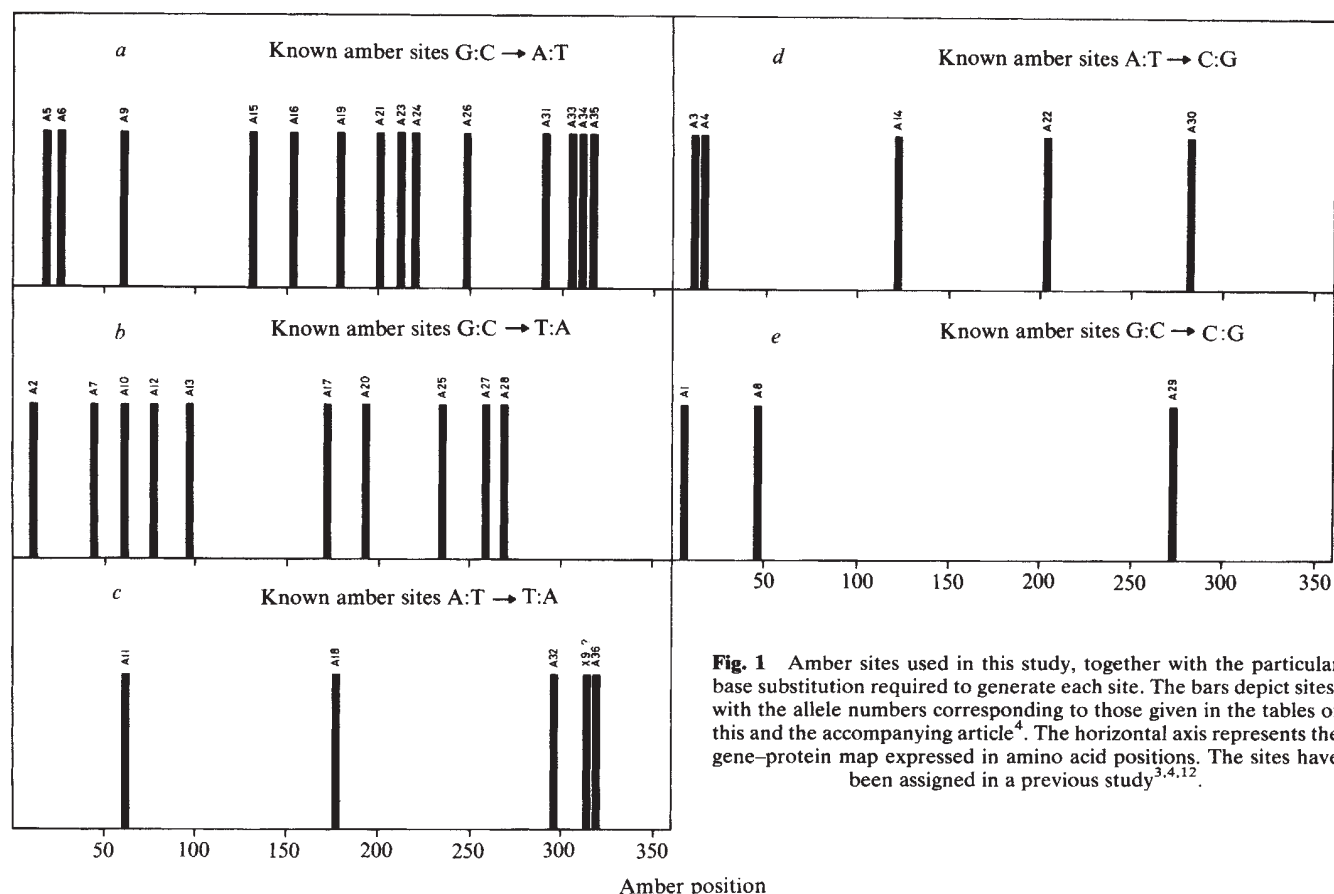
## Spontaneous hotspots

Figures 1 and 2 depict the nonsense mutations which arise through a single base change from wild-type, listing the sites according to the type of base substitution involved. In order to

**Table 1** Spontaneous amber and ochre mutations

| Site | No. of occurrences | Nucleotide sequence |    |
|------|--------------------|---------------------|----|
|      |                    | 5'                  | 3' |
| A5   | 0                  | A T C A G A         |    |
| A6   | 51                 | A C C* A G G        |    |
| A9   | 8                  | A A C A G T         |    |
| A15  | 37                 | A C C* A G G        |    |
| A16  | 9                  | A C C A G A         |    |
| A19  | 11                 | A C C A G C         |    |
| A21  | 8                  | G C T G G C         |    |
| A23  | 8                  | T T C A G C         |    |
| A24  | 10                 | A C T G G A         |    |
| A26  | 6                  | A T C A G A         |    |
| A31  | 1                  | A A C A G G         |    |
| A33  | 5                  | C T C A G G         |    |
| A34  | 12                 | G C C* A G G        |    |
| A35  | 1                  | A T C A G C         |    |
| O9   | 2                  | A A C A A C         |    |
| O10  | 7                  | C A C A A C         |    |
| O11  | 2                  | C G C A A A         |    |
| O13  | 1                  | A T C A A C         |    |
| O17  | 3                  | C G C A A C         |    |
| O21  | 4                  | A G C A A A         |    |
| O24  | 0                  | C G C A A T         |    |
| O27  | 0                  | T T C A A C         |    |
| O28  | 2                  | A A C A A A         |    |
| O29  | 5                  | T G C A A A         |    |
| O34  | 2                  | G G C A A A         |    |
| O35  | 4                  | T G C A A C         |    |

The nucleotide sequence<sup>5</sup> surrounding each base (italic print) that generates a nonsense codon by a transition is shown. The anti-sense strand is depicted. Asterisks (\*) indicate 5-methylcytosine. The number of occurrences refer to the amber and ochre mutations found spontaneously in a collection of 222 amber *I<sup>-</sup>* and 76 ochre *I<sup>-</sup>* mutations. The characterisation of these mutations has been described previously<sup>2,3,12</sup>. Only the transition sites are shown here. Table 2 lists the transversions found among the amber and ochre mutations from this collection.



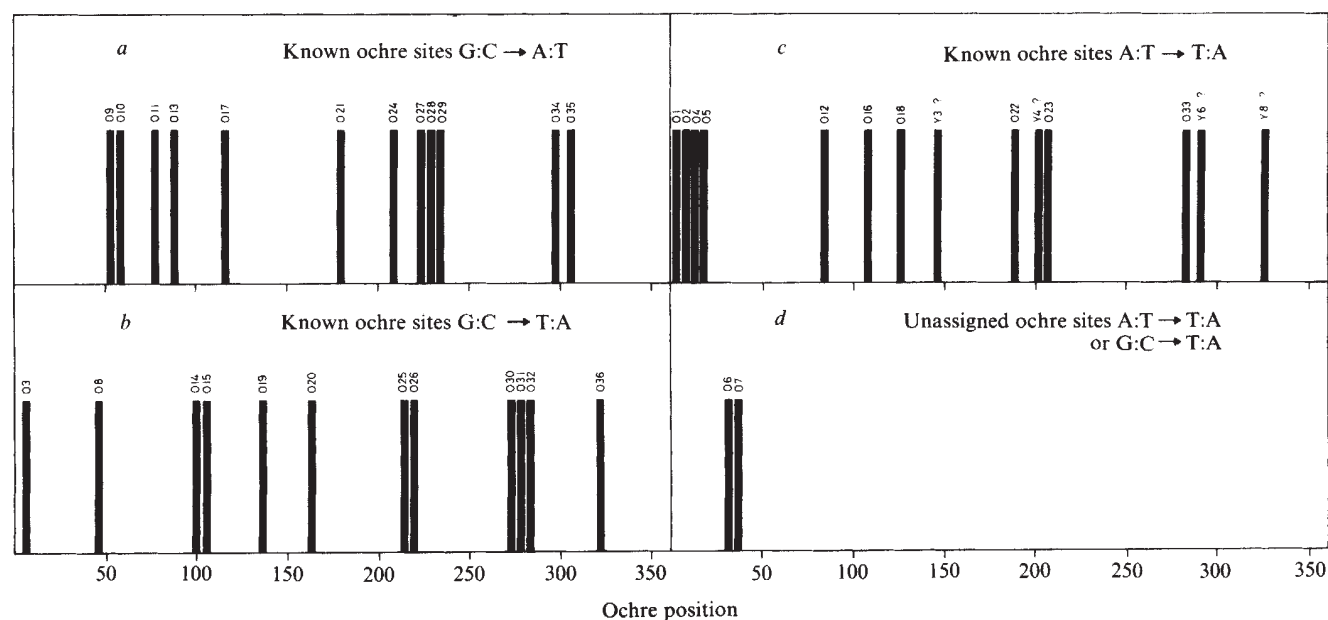
**Fig. 1** Amber sites used in this study, together with the particular base substitution required to generate each site. The bars depict sites, with the allele numbers corresponding to those given in the tables of this and the accompanying article<sup>4</sup>. The horizontal axis represents the gene-protein map expressed in amino acid positions. The sites have been assigned in a previous study<sup>3,4,12</sup>.

determine the frequency of mutations at each site, we picked independent occurrences of *I* mutations, without any selection bias, and assigned them to one of the known sites. Figure 3 shows the relative frequencies of spontaneous amber and ochre mutations. Although amber and ochre mutations arise at similar frequencies, more amber mutations (222) were analysed than ochre sites (76), so we have magnified the ochre scale by a factor of three to allow a direct comparison with the amber bar heights. The amber spectrum is dominated by two

hotspots, both involving a G:C → A:T transition. Out of approximately 70 single base substitutions that generate nonsense codons, 26 of which involve the G:C → A:T transition, mutations at these two sites are favoured by nearly a 10:1 ratio (over the average frequencies at the remaining transition sites).

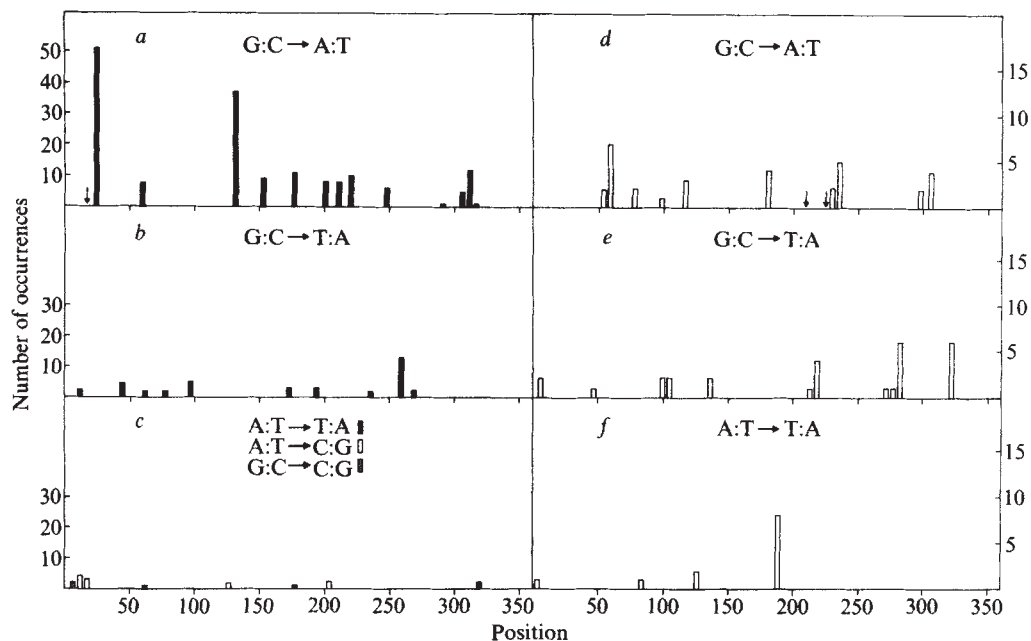
Table 1 lists the sequences surrounding each G:C → A:T transition site. Both hotspots occur at the second C (cytosine) of a CCAGG sequence. One other CCAGG sequence appears

**Fig. 2** Ochre sites resulting from different base substitutions. (See legend to Fig. 1).





**Fig. 3** The distribution of 222 spontaneous amber mutations (*a*, *b*, *c*) and 76 spontaneous ochre mutations (*d*, *e*, *f*). The mutations are of independent origin and have been characterised previously<sup>2,3,12</sup>. Although the ochre scale has been magnified to enable a direct comparison of amber and ochre mutations, hotspots can be detected with a greater degree of assurance by considering the amber and ochre distributions as separate groups. The arrows in *a* and *d* indicate transition sites not found in this collection. (Only half of the spontaneous collection was assayed for the missing site, A5.) Transition sites not represented in this collection can be recognised by consulting Figs 1 and 2.



among the remaining 24 transition sites, but mutations here are not as frequent. Because *E. coli* K12 carries the chromosomal *mec* gene<sup>8</sup>, this sequence, the normal cutting site for the RII restriction endonuclease<sup>9</sup>, should be methylated at the second C from the 5'-end of each strand, generating 5-methylcytosine (C-5meC-A- G-G-). This suggests that 5-methylcytosine may be the cause of the spontaneous hotspots. The presence of 5-methylcytosine at these positions in the *I* gene DNA was verified directly by chemical DNA sequencing<sup>7</sup>, as this substituted residue leaves a characteristic gap in the sequence, with a G (guanine) residue appearing at the corresponding position on the complementary strand.

### Creation of a new CCAGG sequence

To test whether the appearance of hotspots at two of the methylated bases was fortuitous, we introduced a new CCAGG sequence into the gene. The amber mutation A28 is derived from a codon normally specifying serine 269 (refs 4, 5) which lies in the sequence CTCGG, changed to CTAGG in A28. Reversion to a sense codon by a transition would generate either CCAGG or CTGGG, specifying glutamine or tryptophan, respectively. Tryptophan at this site should not produce an active repressor, as leucine and tyrosine, introduced in place of serine by nonsense suppressors produce *i*<sup>-</sup> proteins<sup>4</sup>; while the replacement of serine by glutamine results in a temperature sensitive repressor<sup>4</sup>. We used 2-AP to induce *i*<sup>+</sup> revertants. As all these revertants displayed temperature sensitive repression, they most probably represented the conversion of the amber UAG triplet to a CAG codon. We proved the construction by crossing the mutation onto a plasmid and sequencing the relevant region. We found a new 5-methylcytosine in the CCAGG sequence that replaced the original CTCGG. Figure 4 summarises the steps involved in this construction.

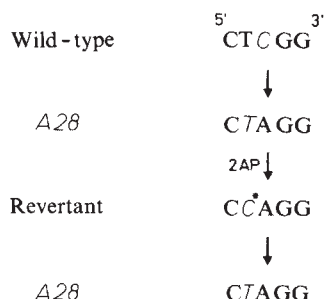
### Amber mutations arising from the new CCAGG sequence

We collected spontaneous *i*<sup>-</sup> amber mutations using an episome carrying the new CCAGG sequence at position 269, in our standard strain background<sup>3</sup>. Figure 5 presents the new distribution of amber mutations. In addition to the two original hotspots, a third hotspot appears at the new CCAGG sequence.

**Table 2** Amber mutations detected in *E. coli* B and K12

| Base substitution | Site | Number of occurrences |          |          |          |
|-------------------|------|-----------------------|----------|----------|----------|
|                   |      | <i>E. coli</i> B      | <i>a</i> | <i>b</i> | <i>c</i> |
| G:C → A:T         | A5   | 3                     | 0        | 0        | 0        |
|                   | A6   | 4                     | 51       | 13       | 64       |
|                   | A9   | 3                     | 8        | 2        | 10       |
|                   | A15  | 3                     | 37       | 15       | 52       |
|                   | A16  | 1                     | 9        | 0        | 9        |
|                   | A19  | 5                     | 11       | 0        | 11       |
|                   | A21  | 6                     | 8        | 0        | 8        |
|                   | A23  | 0                     | 8        | 0        | 8        |
|                   | A24  | 4                     | 10       | 3        | 13       |
|                   | A26  | 0                     | 6        | 0        | 6        |
|                   | A31  | 1                     | 1        | 1        | 2        |
|                   | A33  | 2                     | 5        | 0        | 5        |
|                   | A34  | 3                     | 12       | 5        | 17       |
|                   | A35  | 3                     | 1        | 0        | 1        |
|                   | A28* | —                     | —        | 11       | —        |
| G:C → T:A         | A2   | 0                     | 2        | 0        | 2        |
|                   | A7   | 2                     | 4        | 0        | 4        |
|                   | A10  | 0                     | 2        | 0        | 2        |
|                   | A12  | 0                     | 2        | 0        | 2        |
|                   | A13  | 2                     | 4        | 0        | 4        |
|                   | A17  | 2                     | 3        | 0        | 3        |
|                   | A20  | 1                     | 3        | 2        | 5        |
|                   | A25  | 0                     | 2        | 0        | 2        |
|                   | A27  | 5                     | 13       | 0        | 13       |
| A:T → T:A         | A28  | 2                     | 2        | —        | —        |
|                   | A11  | 0                     | 1        | 0        | 1        |
|                   | A18  | 4                     | 1        | 0        | 1        |
|                   | A32  | 0                     | 0        | 0        | 0        |
|                   | X9   | 0                     | 0        | 0        | 0        |
| A:T → C:G         | A36  | 3                     | 2        | 0        | 2        |
|                   | A3   | 0                     | 4        | 0        | 4        |
|                   | A4   | 0                     | 3        | 0        | 3        |
|                   | A14  | 1                     | 2        | 0        | 2        |
|                   | A22  | 0                     | 2        | 0        | 2        |
| G:C → C:G         | A30  | 0                     | 0        | 1        | 1        |
|                   | A1   | 0                     | 2        | 0        | 2        |
|                   | A8   | 1                     | 0        | 0        | 0        |
|                   | A29  | 2                     | 0        | 0        | 0        |

The amber mutations found spontaneously in *E. coli* B are compared with those found in *E. coli* K12. *a*, The collection in K12 using the wild-type episome from strain GM1 (ref. 12); *b*, that for the episome carrying the additional CCAGG sequence; *c*, combined distribution showing both of these collections together (except for site A28, which is derived by a transition in *b* but by a transversion in *a*).



**Fig. 4** The nucleotide sequence surrounding the codon at position 269. The amber mutation A28 is generated by a C→A transversion from this sequence<sup>3,4</sup>. The resulting CTAGG sequence can be converted by 2-AP to a CCAGG sequence, which is methylated at the second cytosine (marked by asterisk; see text). This restores wild-type activity at 37°C and allows the generation of A28 by a C→T transition at the 5-methylcytosine.

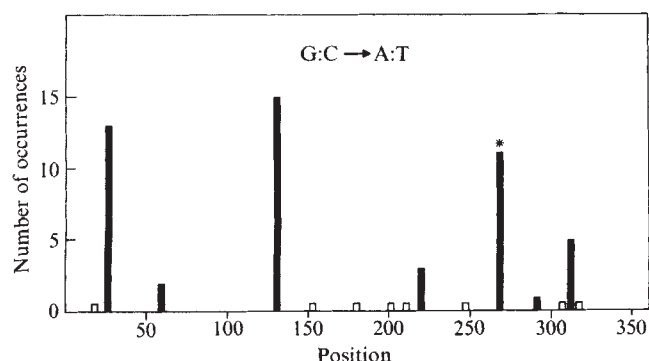
## Strains lacking methylation activity

*E. coli* B strains do not methylate the CCAGG sequence<sup>10</sup>. We crossed the *lacproB* deletion, XIII, into an *E. coli* B derivative in such a manner as to avoid transferring the region of the chromosome carrying the *mec* locus. We checked this by transforming this strain with a plasmid carrying the wild-type *I* gene<sup>11</sup> and sequencing one of the CCAGG sites in the *I* gene. It had unmethylated cytosines. After introducing the *lacpro* episome from strain GM1 (ref. 12) into this *E. coli* B strain, we derived and analysed amber mutations on the episome. Table 2 compares these results with those from the K12 strain. The two hotspots disappear when the *E. coli* B derivative is used. (This is particularly evident when the K12 results obtained with the altered sequence at position 269 are considered, see column *b*, since a similar sample size was analysed). Thus, the spontaneous hotspots are the result of methylation of cytosine at the 5 position.

## 2-Aminopurine induced mutations

The base analogue 2-AP specifically induces transitions at frequencies more than 100-fold above the spontaneous background<sup>12-14</sup>. Figure 6 shows the distribution of over 1,000 ochre and amber mutations induced by 2-AP (Fig. 6a and b, respectively), all arising by the G:C→A:T transition. There are significant hotspots at both amber and ochre sites. The three major amber hotspots are at the CCAGG sequences discussed above, and when we rederived the 2-AP spectrum

**Fig. 5** The distribution of 50 spontaneous amber mutations derived from a G:C→A:T transition. This collection was obtained using a derivative carrying an *F*<sup>+</sup>*lacpro* episome with an additional CCAGG sequence in the *lacI* gene (see text). The asterisk marks the position of amber mutations derived from this sequence. In addition to the mutations shown above, three amber mutations generated by transversions were also found (see Table 2).



using the episome carrying the additional CCAGG sequence at 269, a fourth hotspot appeared at the new position (Fig. 6c). Therefore, 2-AP mutagenesis also favours the methylation CCAGG sequence. However, in the *E. coli* B derivative which lacks the *mec* system that methylates the CCAGG stretches, the 2-AP amber hotspots are reduced but not eliminated. In *E. coli* K12 the two major hotspots are about 27 times more frequent than the average of the other sites; in *E. coli* B this ratio falls by a factor of 4 to 6.7:1. Both the methylation and the sequence seem to affect 2-AP mutability.

None of the ochre sites involves wild-type codons which are part of a methylation sequence, and yet there is still a wide

**Table 3** Nucleotide sequence surrounding each mutational site

| Site | Sequence (5'-3')                                               | No. of occurrences |     |    |    |     |
|------|----------------------------------------------------------------|--------------------|-----|----|----|-----|
|      |                                                                | 2-AP               | EMS | NG | UV | NQO |
| A6   | G A A C <sup>*</sup> A G G C<br>C T T G G T <sup>*</sup> C C G | 270                | 14  | 20 | 15 | 61  |
| A15  | T G A C <sup>*</sup> A G G A<br>A C T G G T <sup>*</sup> C C T | 207                | 7   | 4  | 7  | 54  |
| A34  | G G G C <sup>*</sup> A G G C<br>C C C G G T <sup>*</sup> C C G | 66                 | 15  | 12 | 1  | 55  |
| A9   | C A A A C A G T C<br>G T T T G T' C A G                        | 8                  | 30  | 34 | 1  | 61  |
| A31  | C A A A C A G G A<br>G T T T G T C C T                         | 9                  | 19  | 18 | 2  | 20  |
| A16  | T G A C C A G A C<br>A C T G G T C T G                         | 11                 | 14  | 21 | 20 | 29  |
| A19  | T C A C C A G C A<br>A G T G G T C G T                         | 23                 | 30  | 37 | 16 | 13  |
| A21  | A T G C C A G C C<br>T A C G G T C G G                         | 15                 | 12  | 22 | 5  | 16  |
| A24  | A C T C C A G T C<br>T G A G G T C A G                         | 13                 | 39  | 34 | 9  | 51  |
| A5   | T T A T C A G A C<br>A A T A G T C T G                         | 3                  | 17  | 13 | 19 | 23  |
| A23  | A A T T C A G C C<br>T T A A G T C G G                         | 2                  | 30  | 32 | 80 | 24  |
| A26  | C G C T C A G A T<br>G C G A G T C T A                         | 5                  | 20  | 12 | 2  | 36  |
| A33  | C T C T C A G G G<br>G A G A G T C C C                         | 7                  | 38  | 24 | 36 | 33  |
| A35  | C A A T C A G C T<br>G T T A G T C G A                         | 1                  | 27  | 9  | 6  | 25  |
| O11  | G T C G C A A A T<br>C A G C G T T T A                         | 41                 | 56  | 28 | 2  | 11  |
| O17  | C G C G C A A C G<br>G C G C G T T G C                         | 44                 | 27  | 26 | 3  | 28  |
| O21  | C C A G C A A A T<br>G G T C G T T T A                         | 26                 | 57  | 15 | 2  | 43  |
| O29  | C A T G C A A A T<br>G T A C G T T T A                         | 77                 | 36  | 14 | 4  | 83  |
| O34  | G G G G C A A A C<br>C C C C G T T T G                         | 112                | 46  | 19 | 2  | 47  |
| O35  | G C T G C A A C T<br>C G A C G T T G A                         | 81                 | 49  | 22 | 3  | 48  |
| O9   | G G C A C A A C A<br>C C G T G T T G T                         | 3                  | 23  | 18 | 3  | 15  |
| O10  | A C A A C A A C T<br>T G T T G T T G A                         | 11                 | 17  | 10 | 4  | 31  |
| O28  | T C A A C A A C A<br>A G T T G T T T G                         | 11                 | 13  | 12 | 4  | 24  |
| O13  | C A A T C A A A T<br>G C T A G T T G A                         | 5                  | 13  | 9  | 6  | 37  |
| O24  | C A A T C A A A T<br>G T T A G T T T A                         | 2                  | 16  | 16 | 61 | 22  |
| O27  | T T T T C A A C A<br>A A A A G T T G T                         | 5                  | 15  | 20 | 86 | 10  |

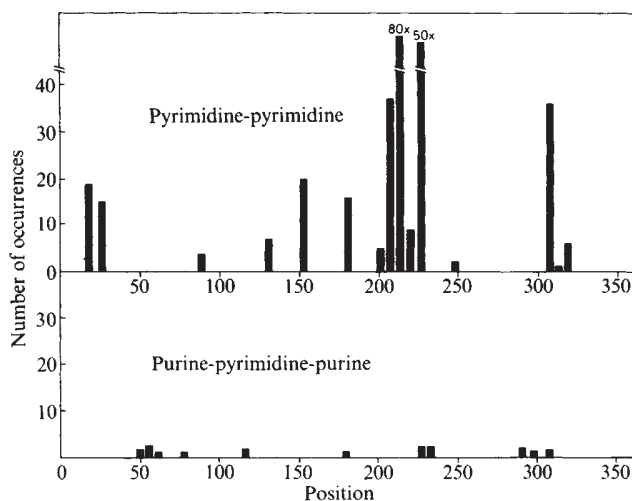
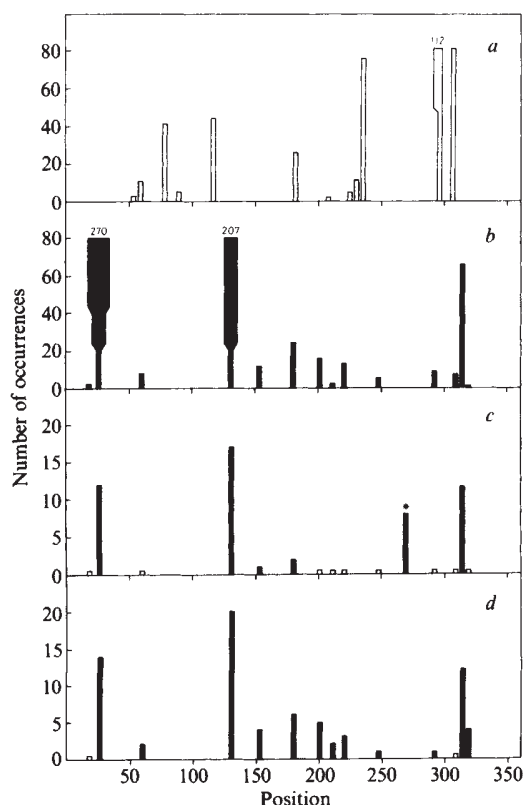
variation in the frequency of the G:C→A:T transition at these points. Table 3 shows the DNA sequence surrounding each mutational site. As described below, the C in a GCA sequence is more highly mutable with 2-AP.

### Effect of neighbouring nucleotide sequence on mutagen-induced mutation rates

In Table 3 we present an analysis of the nucleotide sequence surrounding each mutational site which generates an amber (UAG) or ochre (UAA) codon by a G:C→A:T transition. The base involved in the transition is italicised. The columns give the relative frequencies of occurrence of each amber or ochre site in a collection of mutagen induced  $I^-$  mutations. One amber and one ochre mutation for each mutagenised culture was analysed<sup>1,2</sup>, and therefore each mutation is of independent origin. A correction factor is necessary if amber and ochre mutations are to be considered together, as these two types of mutation did not always arise at identical frequencies, and different sample sizes were analysed. To compare 2-AP-induced ochres with the 2-AP-induced amber mutations, the ochre values should be multiplied by 0.9. For 4-nitroquinoline-1-oxide (NQO), ultraviolet light, ethyl methanesulphonate (EMS) and N'-methyl-N'-nitro-N-nitrosoguanidine (NG) ochres the respective factors are 0.6, 0.7, 2.3 and 1.9.

Even without placing too much emphasis on normalising amber and ochre frequencies, some patterns seem to emerge from these data. 2-aminopurine clearly prefers methylated cytosines for the G:C→A:T transition (see ref. 4). Otherwise,

**Fig. 6** Distributions of 418 ochre (a) and 640 amber mutations (b) induced by 2-AP. These mutations were characterised in a previous study<sup>12</sup>. Fifty-two amber mutations were derived by 2-AP using the episome carrying an additional CCAGG sequence surrounding position 269 (see text). c, The distribution of these mutations. The asterisk (\*) marks the position of site A28 which is derived by a C→T transition at the new CCAGG sequence. 2-AP-induced amber mutations derived in *E. coli* B are represented in d. The open bars in c and d refer to amber sites derived by C→T transitions which are not represented in these particular collections.



**Fig. 7** Correlation of UV-induced mutations with the surrounding nucleotide sequence (see text).

the different frequencies of occurrence do follow the nature of the base on the 5' side of the cytosine residue, the tendency being G>C>A>T.

Figure 7 shows a strong correlation between being well induced by ultraviolet and being part of a pyrimidine-pyrimidine sequence (on one strand or the other), and a strong correlation for being poorly induced and not being part of such a sequence. All of the amber sites are in the pyrimidine-pyrimidine category except for A9 and A31, whereas only the ochre sites O13, O24, and O27 are part of a pyrimidine-pyrimidine sequence.

The EMS, NG and NQO data do not seem to allow any general rules to be deduced at this stage.

### Hotspots involve methylated cytosines

By using a set of characterised nonsense mutations and the full nucleotide sequence of the *lacI* gene, we have demonstrated that base substitution hotspots occur at 5-methylcytosines. Two of the three methylated cytosines, which occur among 26 cytosines involved in the creation of amber and ochre codons, generate transitions at a 10-fold higher rate than the average. A new methylated cytosine introduced by creating an additional CCAGG sequence also results in a hotspot for the G:C→A:T transition. The elimination of methylation at these sites, demonstrated in an *E. coli* B derivative lacking the methylating enzyme, results in the disappearance of the transition hotspots.

What mechanism could account for these results? 5-methylcytosine does not cause any direct mispairing<sup>15</sup>, nor do *in vitro* measurements indicate a significant difference between cytosine and 5-methylcytosine in the tautomeric equilibria<sup>16</sup>. An attractive explanation is the deamination of cytosine to uracil, which occurs spontaneously at a high rate *in vitro* and presumably *in vivo*<sup>17</sup>. Uracil residues in DNA are rapidly excised by the enzyme uracil-DNA glycosylase<sup>18</sup>. Any uracil residues which are not removed would eventually result in G:C→A:T transitions, unless otherwise repaired. (Drake and coworkers have shown that spontaneous transition mutations occur in T4 DNA on extended storage in the cold, as a result of deamination of cytosine<sup>17</sup>. T4 DNA contains glucosylated 5-hydroxymethylcytosine instead of unsubstituted cytosine; after deamination, the resulting 5-hydroxymethyluracil residues are not subject to the action of uracil-DNA glycosylase, probably accounting for the high rate of transition mutations.) Although most spontaneous deaminations of cytosine to uracil would be efficiently repaired by uracil-DNA glycosylase, those occurring at 5-methylcytosine residues would yield thymine (5-methyluracil), which will not be released from DNA by this enzyme.



The G:T base pair would still be subject to various types of mismatch repair, as would any G:U base pairs which escape the action of the glycosidase. The greatly increased number of unexcised G:T base pairs which originated from G:5meC would then appear as a hotspot relative to other transition sites. This argues that deamination is a major cause of spontaneous transitions in *E. coli*, at least in the direction G:C→A:T. This model predicts that these hotspots should disappear in mutant strains lacking uracil-DNA glycosidase, since deaminations at all cytosines should then be immune to this excision repair. Such mutants, termed Ung<sup>-</sup>, have been described<sup>20</sup>. Hotspots among nitrous acid-induced amber mutations should also occur at 5-methylcytosines, since this mutagen preferentially stimulates deamination of cytosine<sup>15</sup>.

Thus T is used in place of U in DNA to lower the mutation rate, the role of the methyl group being to distinguish one natural base from the deamination product of the other<sup>18</sup>. Possibly the structures of A and G are also selected to allow enzymatic repair of deamination.

The base analogue 2-AP shows a remarkable selectivity for certain sites. All three CCAGG sequences are hotspots for amber mutations, and a fourth CCAGG sequence introduced into the gene results in the appearance of a new hotspot. However, the elimination of methylation at these sites does not entirely remove the hotspots, although it does reduce their relative intensity by a factor of 4. One possibility is that a residual methylation exists in these strains, too low to detect by our methods. Alternatively, a different enzyme in the cell could be operating on these sites, or some subtle aspect of the base sequence may be responsible for the high mutability in the presence of 2-AP. The same situation is seen for the 2-AP-induced ochre mutations, where certain unmethylated sequences clearly result in significant hotspots. The elucidation of the mechanism that produces this phenomenon requires additional experiments. Other mutagens, such as ultraviolet light, NQO, NG, and EMS also result in considerable differences in mutation rate from site to site. The hotspots

favoured by these mutagens, particularly ultraviolet light, are different from those described above. At least for 2-AP some rules are evident. Higher mutation rates occur when G is the adjacent base, in accordance with both predictions and *in vitro* findings of M. Bessman and coworkers (personal communication).

We thank S. Brenner, S. Hattman, W. Arber, J. D. Watson, M. Radman, T. Lindahl and A. Maxam for helpful discussions, and M. Hofer for technical assistance. This work was supported by a grant from the Swiss National Fund (3.179.77) to J.H.M. and an NIGMS grant to W.G. W.G. is an American Cancer Society Professor of Molecular Biology.

**Note added in proof:** Experiments with this system (B. K. Duncan and J. H. Miller, unpublished) show that G:C→A:T transitions occur at a high rate in an Ung<sup>-</sup> strain, and that the 5-methylcytosine residues are no longer hotspots relative to the other cytosines.

Received 27 February; accepted 26 June, 1978.

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# letters to nature

## Detecting nonsolar planets by spinning infrared interferometer

THE only known planets are the nine in our Solar System, so the origin of the Solar System and of stars in general would be clarified if other planetary systems could be discovered. Likewise, speculation about life in the Galaxy would benefit from knowing which stars have planets. Astrometry, radial velocity measurement, and direct photography from orbit might detect planets, but none of these approaches will be easy. Astrometry would require detection of the motion of a stellar image, relative to background stars, by  $\pm 0.5$  arc ms as 12 yr elapse (for a system identical to the Sun plus Jupiter but situated at a distance of 10 pc). Alternatively, a radial velocity change of  $\pm 12 \text{ m s}^{-1}$  over the 12 yr would reveal a Jupiter-like planet which would mean detecting a spectral line shift of  $2 \times 10^{-4}$  line widths in a line 0.1 nm wide. The instruments required would need stability over a period of years as well as sensitivity. Direct photography from orbit would invoke precision apodisation and would require surface accuracies on optical surfaces of hitherto unattained quality. The new infrared method proposed here also needs to be considered.

Reference data for the standard system are as follows (planet Jupiter in parentheses). Temperature 6,000 K (128 K), angular diameter  $4.7 \times 10^{-9}$  ( $4.7 \times 10^{-10}$ ), distance  $3 \times 10^{17}$  m, angular separation  $2.6 \times 10^{-6}$ . At maximum elongation, the visible

planetary light is  $2 \times 10^{-9}$  of the starlight, assuming an albedo of 0.2. In the Rayleigh-Jeans regime, however, the planetary infrared power, if simplifying assumptions are made about emissivities and limb darkening, is  $2 \times 10^{-4}$  of that from the star. Thus a factor of  $10^5$  favours the infrared.

At a wavelength just under  $10 \mu\text{m}$  the advantage is eliminated and choice of wavelength must be to the long wavelength side of about  $20 \mu\text{m}$ .

A way to enhance the planet over the star is to place an interference null on the star. An interference pattern  $\sin^2(\pi\theta/\Phi)$  has a maximum on a planet at  $\theta = \Phi/2$  when response to a star at  $\theta = 0$  is minimised. The necessary interferometer baseline at  $40 \mu\text{m}$  is 7.7 m. Ignoring stellar limb darkening we find that the planet-to-star power ratio at  $40 \mu\text{m}$  is 62 when a null is centred on the star and the planet is on an interference maximum.

Where small signals have to be detected in the presence of unwanted signals one modulates the desired signal. If the interferometer spins with frequency  $\omega$  about an axis through the star, the stellar signal will not vary but the planetary signal will vary as  $\sin^2[(\pi/2)\cos\omega t]$ , a function that contains a noticeable amount (31%) of the  $4\omega$ -harmonic. Very faint signals can be recovered if they are modulated at a known frequency by synchronous detection that selects that frequency. Alternatively, where the modulation is substantially non-sinusoidal, the received signal can be broken into one-period segments and averaged.

In the presence of pointing error the star power will vary but not in the same way as the planetary signal. The planetary modulation will be markedly different if the planet is  $\kappa$  fringe spacings away instead of half a spacing. The  $4\omega$ -content of  $\sin^2[(\kappa\pi)\cos\omega t]$  is distinctly enhanced; in fact the fundamental at  $2\omega$  can be suppressed completely. On the other hand, the star signal will be predominantly at  $2\omega$ . Hence, singling out the  $4\omega$  component allows us to relax the pointing accuracy. Further aid in disentangling star from planet could come from choosing two wavelengths to capitalise on the differences in spectra of star and planet.

Stability of 1 arc ms, or about the angular diameter of the star, should be achievable using a massive rotating body in space. The most obvious approach would be continuous attitude control based on an optical star tracking telescope as in the satellite-borne gyroscopic test of general relativity<sup>1</sup>. The telescopic axis would be fixed on the interferometer null and share as much as possible the infrared optics.

It is reasonable to assume that, in the future, infrared detectors will not be limiting. Even then, the optics will be cooled by liquid helium and shielded from terrestrial radiation as in planned infrared telescopes<sup>2,3</sup>. With  $\Delta\lambda/\lambda = 0.1$ , about two planetary photons will be collected per  $\text{m}^2$  per s. But the random arrival will produce photon noise. To make a reading to 10% accuracy with  $1 \text{ m}^2$  of collector would take 100 s. The unavoidable photon noise would determine the detection time were it not for infrared emission from the particles, about  $1 \mu\text{m}$  in size and about 1 km apart, that contribute to the zodiacal light. Rocket observations reported<sup>4,5</sup> in the range  $5\text{--}23 \mu\text{m}$  are fitted by a grey-body emission spectrum corresponding to a colour temperature of 280 K and an optical thickness of  $3 \times 10^{-8}$ . This means a photon flux density of  $6 \times 10^8 \text{ photons s}^{-1} \text{ cm}^{-2} \mu\text{m}^{-1} \text{ sr}^{-1}$ . With the numerical data adopted, integration over one month would reduce the r.m.s. fluctuation in zodiacal light to  $3.5 \times 10^5$  photons permitting the  $5 \times 10^6$  planetary photons to be detected. Because the zodiacal particle density falls off rapidly with distance from the Sun and from the ecliptic plane, the infrared technique would be appropriate for planned missions to Jupiter and out of the ecliptic plane.

Meanwhile little is known about our infrared environment and the rocket observations cited might contain an avoidable contribution from the Earth's atmosphere. A sensitive infrared sky survey is needed to improve our knowledge of the zodiacal emission because all we have now is data from the limited arc swept out in the rocket work. The planned infrared astronomical satellite will be sensitive enough to detect the zodiacal emission and to determine whether the intensity in the  $40\text{--}60 \mu\text{m}$  range follows the grey-body extrapolation. Lack of knowledge about possible galactic and extragalactic contributions and about the nature of the zodiacal particles makes extrapolation uncertain at present.

The resolution and sensitivity of the spinning infrared interferometer suggest other applications, for example, to cool features other than planets (such as dust rings or substellar companions). The sensitivity is capable of enhancement by enlargement of the collecting area or by attention to stars closer than 10 pc.

In developing this concept I benefited from NASA workshops: 'Search for Extraterrestrial Life' and 'Extrasolar Planetary Detection' and a NASA summer course.

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Received 14 April; accepted 9 June 1978.

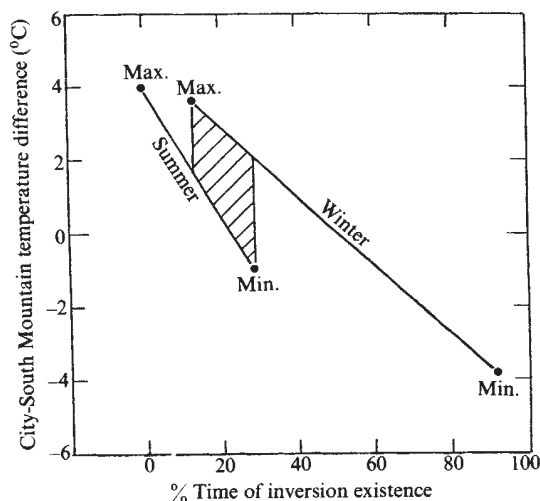
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## Climatological effects of atmospheric particulate pollution

THE large amount of dust thrown into the atmosphere by volcanoes has long been recognised as an important factor in initiating climatic change that generally leads to cooler surface temperatures<sup>1-4</sup>. Another source of atmospheric dust is man's agricultural and industrial activity. It has been suggested that this anthropogenic source is a cause of cooling in a manner similar to that of volcanoes<sup>5,6</sup>. The primary mechanism thought to be responsible for cooling in both these situations is the backscatter to space of a portion of the incoming solar radiation. This reduction in heat input to the planet would cool its surface. Idso<sup>7-10</sup> has argued for another effect of atmospheric dust—warming due to 'thermal blanketing'. This mechanism would absorb some of the Earth's thermal radiation that would otherwise escape to space and then reradiate a portion of this radiation back to the surface, hence a rise in surface temperature with increasing atmospheric dust would be expected. We have completed an experiment recently in which the relative magnitudes of the solar and thermal radiation interactions with atmospheric dust were compared<sup>11</sup>. The results reported there indicate that there is a general climatological superiority for the thermal radiation interaction, predicting a net warming trend with increasing atmospheric dust for all but the most extreme cases of dust-loading of the atmosphere.

We therefore have a major apparent contradiction; our experiments predict warming with increasing atmospheric dust but most studies of climatic trends following periods of significant volcanism indicate cooling. These divergent results can be explained by the concept of a 'centre of gravity' for atmospheric thermal emission, loosely defined as the atmospheric level below which half of the thermal radiation that reaches the surface originates. As previous studies<sup>12,13</sup> indicate that this level is only of the order of a few hundreds of metres, volcanic eruptions that inject dust several thousands of metres into the stratosphere may have relatively little effect on the thermal radiation received at the surface. Thus, the solar radiation interaction probably predominates in such situations, with resultant cooling. Since all of the dust-loading situations which we investigated were confined to the lower troposphere, we have characteristically observed a tendency for warming. This is the region of the atmosphere affected by man's activities

**Fig. 1** The temperature difference between the base level of the city of Phoenix, Arizona, and the top of adjacent South Mountain (450 m higher) plotted against the percentage time of inversion existence at the times of maximum and minimum temperature measurements in winter and summer.





so we would also expect a tendency for warming following major anthropogenic activity.

We looked for an experimental situation that might confirm our findings. Fortunately, our location in the centre of metropolitan Phoenix, Arizona, was well suited to the investigation of our concept; it has a topographic disturbance (South Mountain) on its outer periphery that rises about 450 m above the base of the city proper, rather near the centre of gravity for atmospheric thermal emission.

The data we used in our analysis were standard maximum and minimum air temperatures obtained from the National Weather Service station at the city centre and a cooperating station at the top of South Mountain. For all cloudless days between 1974 and the present, we calculated the mean temperature differences between the 450-m altitude differential provided by these two stations for maximum and minimum temperatures in summer (May to August; 185 days in total) and winter (November to February; 205 days in total). Then, from data describing inversion characteristics of the atmosphere over Phoenix<sup>14</sup>, we calculated the per cent time that inversions were present at the times of these four temperature measurements (max. winter, max. summer, min. winter, min. summer).

When these temperature differences are plotted as a function of these percentage times of inversion existence (Fig. 1), the primary effect observed is that of the inversion phenomenon itself. That is, for greater percentage times of inversion existence, the city proper becomes progressively cooler than the higher South Mountain station, with a temperature shift of about 8°C for the full range of percentage times of inversion existence shown. However, we see that the summer and winter trend lines are significantly separated from each other, implying a perturbation acting on the primary effect of the inversion phenomenon. To discover the nature and size of this perturbation, we compared the summer and winter trend lines for equivalent percentage times of inversion existence (the cross-hatched area of Fig. 1) finding that the city proper is warmer in winter than in summer, relative to the top of South Mountain, by an average of 2.4°C, once the major inversion effect is removed.

What causes this perturbation? We must first consider that inversions are likely to be more intense in winter than in summer. Any correction for this effect, however, would tend to create an even greater separation between the summer and winter trend lines of Fig. 1. Thus, that factor cannot be the cause of their present separation.

It may at first be suspected that urban heat island effects would be more pronounced in winter than in summer; but the opposite has been found to be true<sup>15-17</sup>. Thus, any adjustment for this effect would also tend to separate the summer and winter trend lines of Fig. 1 even more.

Finally, we consider the thermal blanketing phenomenon and what happens in the absence of inversions. The primary factor that influences the thermal radiative effects of atmospheric pollutants in this situation is the mean seasonal mixing height, or the level to which pollutants can be mixed following breakup of inversions or prevailing in their absence.

For the winter months studied, previous extensive radiosonde data<sup>14</sup> indicated that the mean maximum mixing height was about 750 m above the base level of the city. The major concentration of surface-generated particulates would therefore be expected to be located between the base level of the city and the top of South Mountain at that time of year; and a complete year's study of solar radiation transmittance data<sup>14</sup> indicated that this was so. Consequently, the thermal blanketing phenomenon in winter would be expected to greatly favour increased temperatures at the base level of the city.

In summer, however, the earlier study<sup>14</sup> indicated that surface-generated particulates may be mixed to heights between 2,500 and 3,000 m above the base level of the city, in the absence of inversions. Mixed to heights several times greater than the centre of gravity for atmospheric thermal emission—

even for the South Mountain station—particulates in this season would be expected to exert a much reduced thermal blanketing effect at both the base level of the city and the top of South Mountain. Thus, in summer, neither station would be expected to be favoured with so great a thermal blanketing effect as is the base level of the city in winter; which is what the temperature data of Fig. 1 indicate.

We conclude that the altitudinal location of atmospheric particulates can greatly alter the magnitude of the thermal blanketing effect at the Earth's surface. For natural volcanoes that inject particulates into the stratosphere, the thermal blanketing effect is probably very reduced, with cooling generally resulting. For man's activities which normally inject particulates into the lower troposphere, however, thermal blanketing is very significant; and the most probable tendency for surface temperature change is warming.

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Received 12 April; accepted 19 June 1978.

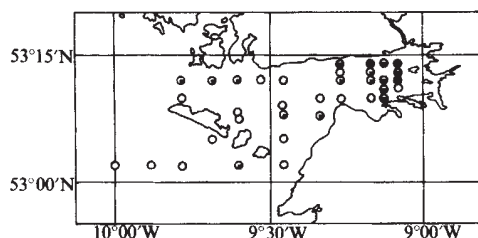
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## Colour, ultraviolet absorbance and salinity of the surface waters off the west coast of Ireland

INTEREST in the relationship between the colour of seawater and various other surface properties of seawater has increased in anticipation of the launch of the Coastal Zone Colour Scanner, a multispectral instrument, aboard Nimbus-G satellite. During the late summer and autumn of 1977, at 73 hydrographic stations in Galway Bay and other waters along the west coast of Ireland (Fig. 1), measurements of water colour and of various chemical, biological and physical properties were obtained in accord with an expanded version of the 'sea-truth' protocol adopted for the 'Ocean Colour Scanner' experiment conducted earlier in the North Sea<sup>1</sup>. The strong correlations found between colour and surface salinity, and between colour and the concentration of dissolved organic matter, suggest that the images to be transmitted from this multispectral scanner will be useful in the identification and delineation of the runoff-influenced waters found along the west coast of Ireland.

Water colour was determined by viewing a white Secchi disk held at one-half its extinction depth, and matching its apparent colour with one of the numerous paint chips on the ocean

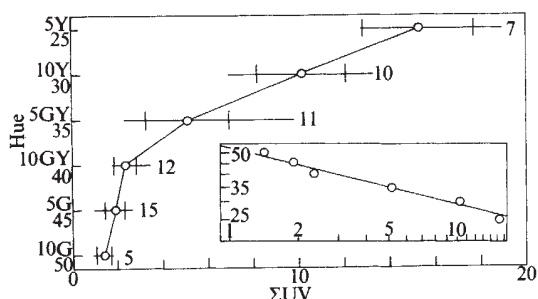




**Fig. 1** Location of 'sea-truth' hydrographic stations in Galway Bay, and in the Atlantic Ocean west of the Aran Islands. ○, Stations occupied once; ●, stations occupied twice; ●, stations occupied three times; ●, stations occupied four times; ●, station occupied five times. Note the Corrib River flowing into the north-east corner of the bay. Five stations not shown on chart are to the south in the Shannon Estuary.

colour scale developed by R. W. Austin of the Scripps Institution of Oceanography. This scale is based on the Munsell system of colour notation, an essentially cylindrical coordinate system with the three colour dimensions being hue, value and chroma<sup>2</sup>.

The angular coordinate is the Munsell hue, hue being that colour attribute whereby red is distinguished from yellow and so on. (The closed circle of Munsell hue is divided into 100 units, which in the preferred notational scheme are collected into 10 major hues, such as yellow-red, with 10 units in each. Thus a particular Munsell hue might be designated as 40, but would more appropriately be designated as 10GY, the tenth unit in the green-yellow major hue.) The axial coordinate is the Munsell value, a measure of the lightness of the colour being described. (The value scale extends from 0 for absolute black, through the neutral greys, to 10 for absolute white.) The remaining, radial, coordinate is the Munsell chroma, which is a measure of the saturation, or departure from neutral grey, of a colour of a particular Munsell value. (The chroma scale begins with 0 for neutral grey and extends out to about 20 for the most-saturated, strongest colours.) A colour with a hue of 10GY, a value of 7, and a chroma of 6, is denoted as 10GY 7/6.

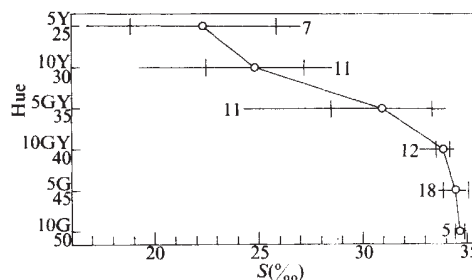


**Fig. 2** Munsell hue plotted against summed UV absorbance, for 60 observations where the Munsell value was 7 and the Munsell chroma was 6. The points are mean summed UV absorbance for all observations of a particular hue. The short vertical bars are one standard deviation on either side of the mean and the absolute range of summed UV absorbance encountered at stations of a given hue is indicated by the extent of the horizontal line. The number of stations of each hue is recorded to the right of each point. Insert: log-log plot of same quantities. Straight line shown has slope of  $-0.258$ .

At 64 of the 73 hydrographic stations the water colour was found to have a value of 7 and a chroma of 6. The remarks below refer only to those stations. The salinity of water samples collected from the first metre beneath the sea surface at each station was determined in the laboratory using an Auto-Lab model 601 MKIII inductively coupled salinometer.

At 60 of the stations surface samples were collected for the determination of UV absorbance. These samples were filtered aboard ship through Whatman GF/C glass fibre filter papers and stored in glass bottles protected from light. Within 1 week, measurements of absorbance were made on these samples at 5 nm intervals between 250 and 350 nm using a Perkin-Elmer 139 UV/visible spectrophotometer fitted with 100 mm cells. The 21 measurements taken on each sample were summed to provide a measure of the concentration of dissolved organic matter<sup>3-5</sup>, in part the Gelbstoff of Kalle<sup>6,7</sup>. The range of wavelengths was chosen to exclude the shorter wavelengths at which nitrate and bromide absorb<sup>8</sup>.

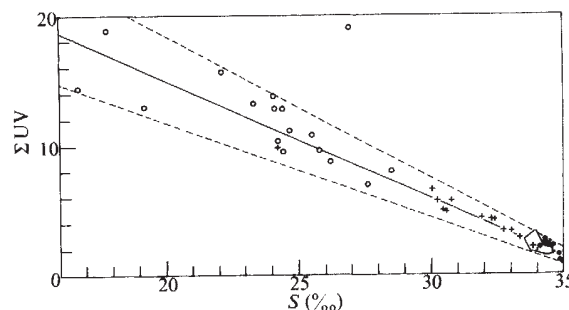
Samples for the determination of summed UV absorbance were also collected from a depth of 10 m, but the comparisons that follow involve only the surface measurements, since at only 11% of the stations were Secchi disk extinction depths as great as 10 metres, so Munsell colour was determined by looking down through a column of seawater extending  $<5$  m beneath the sea surface at 89% of these stations.



**Fig. 3** Munsell hue plotted against surface seawater salinity, for 64 observations where the Munsell value was 7 and the Munsell chroma was 6. The points indicate mean salinity for all observations of a given hue. The short vertical bars are one standard deviation on either side of the mean and the absolute range of surface salinities encountered at stations of a given hue is shown by the extent of the horizontal line. The number of stations of each hue is recorded next to each point.

The expected marked positive correlation between the 'yellowness' of the surface water encountered at a particular station and the summed UV absorbance of the surface sample collected there was observed (Fig. 2). That the less saline samples collected during the latter half of 1977 came from the stations where the surface waters were more yellow in hue is apparent from Fig. 3. These results are consistent with the hypothesis that the yellow matter found in these coastal waters is associated with the humic materials introduced into the sea in the freshwater flowing off the land and out of the streams and rivers.

**Fig. 4** Summed UV absorbance of surface seawater plotted against surface seawater salinity for observations where the Munsell value was 7 and the Munsell chroma was 6. Filled circles, points for stations where Munsell hue of sea water was green. Crosses, points for stations where Munsell hue was green-yellow. Open circles, points for stations where Munsell hue was yellow. Small polygon encloses region of densely packed crosses and filled circles. Solid line defined by equation,  $\Sigma UV = -0.92S + 33.4$ , is mid-line of data envelope marked by dashed lines.



The results presented in Fig. 4, showing summed UV absorbance plotted against surface salinity, with the low absorbance associated with open ocean salinities, suggest that in autumn 1977 *in situ* marine biological activity was at most a minor contributor of the detected organic material. When the mid-line of the data envelope of Fig. 4 is extrapolated back to a salinity of 0‰, it yields a summed UV absorbance of 33 units, which is barely one-half the summed absorbance determined for a sample taken during November 1977 from the major river flowing into Galway Bay. This discrepancy suggests that some alteration of the yellow pigment may be occurring when the freshwater in which it is dissolved begins to mix with saltwater<sup>9</sup>.

It should be noted that the concentration of chlorophyll *a*, as determined for 38 of these stations, was typically low, with the highest concentration encountered being  $1.88 \text{ mg m}^{-3}$ .

Thus, if a satellite equipped with a multi-spectral scanner capable of measuring seawater colour had been passing over the coastal waters west of Ireland during the autumn of 1977, it would have been possible, using 'sea-truth' data of the type reported here, to determine with some degree of accuracy the surface distribution of salinity and dissolved organic matter.

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Received 10 April; accepted 16 June 1978.

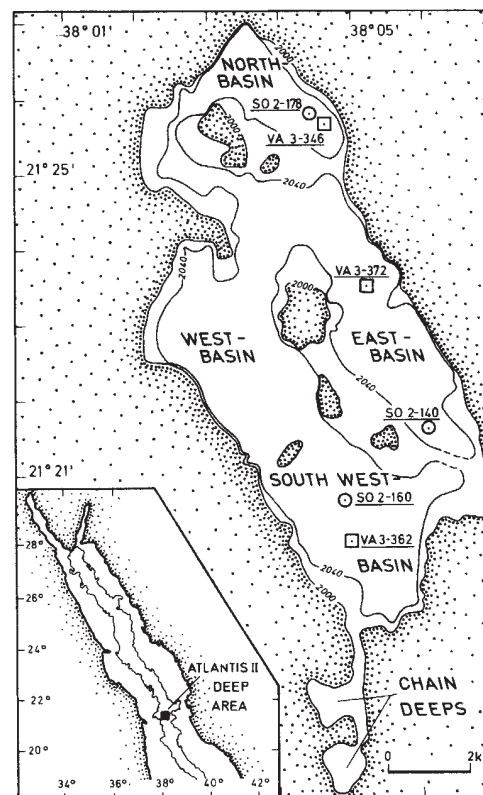
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## Changing hydrothermal activity in the Atlantis II Deep geothermal system

THE Atlantis II Deep is a pool of hot saline brine at the bottom of the central rift in the Red Sea (Fig. 1). Since its discovery in 1965 (ref. 1) it has undergone repeated detailed investigations<sup>2,3</sup>. A permanent increase of temperature has been reported by many workers<sup>4–8</sup> and was interpreted as the result of the injection of hot brine to the system. The temperature of the inflowing brine was estimated to range between 110 and 210 °C (refs 6, 8). We have reinvestigated (after five years) the Atlantis II Deep on board RV Sonne. We report here the results of temperature measurements which were carried out in November 1977.

We used the same instruments as for our previous temperature measurements<sup>7,8</sup>: first, a platinum high resistance thermoprobe as a semicontinuously recording instrument (Bathysonde T 83(3)); and, second, deep-sea reversal thermometers. The reversal thermometers have been calibrated and their absolute accuracy is estimated to be  $\pm 0.1$  °C over the past 5 yr. The bathysonde thermoprobe gave readings of the temperature which were 0.1–0.4 °C higher than those of the deep-sea reversal thermometers at the same position. We, therefore, calibrated all temperatures in Table 1 according to the deep-sea reversal thermometer readings.

The temperature structure of the system is essentially the same as described previously (see Fig. 2). However, temperatures within the two convection layers have changed. The convective mixing within the lower brine brought about a marked increase in the temperature in remote basins of the pool (Table 1). In the North Basin the temperatures are still lower than in the South-west Basin, which indicates that the convective exchange of brine is continuing.



**Fig. 1** Bathymetry of the Atlantis II Deep brine pool. The hydrographic stations which are discussed in this paper are indicated. The 2,000 m isobath is the approximate contour of the top of the mixing layer (ML in Fig. 2) and the 2,040 m isobath contours the top of the lower convection layer.

A significant decrease of the temperature by about 0.7 °C since 1972 can be seen in the upper convection layer (see Table 1). A change of the temperature in this layer, however, is not representative of the whole system and may be caused by mixing from above and advective processes.

The temperature development of the lower convection layer in the South-west Basin, that is, the discharge area of the Atlantis II Deep brine pool should reflect changes in the hydrothermal activity. Table 1 and Fig. 2 show the temperatures to have risen there, again since 1972, by 1.71 °C in the lower convection layer. This increase of temperature is equal to a mean heating rate of only 0.29 °C per year. When compared with the value in 1971–72 of 0.75 °C per year it becomes obvious that the heating rate has slowed down considerably.

An accompanying phenomenon is the change of the temperature structure of the high temperature zone immediately below the interface of the upper and lower convection layer (see insets in Fig. 2, and Table 1). The temperature peaks were previously clearly detectable in this zone. The differences of mean and maximum temperatures in 1971 and 1972 were 0.58 °C and 0.26 °C, respectively. This characteristic structure is at present only detectable with difficulty by the temperature probe; that is no rising hot brine is presently accumulating below the interface. We regard both the decrease of the heating

**Table 1** Temperatures in various depths of the upper- and lower-convection layer (UCL and LCL, respectively) as well as in the high temperature zone (HTZ) below the interface LCL/UCL

| Zone<br>(see Fig. 2) | Depth<br>(dbar) | South-west<br>Basin |                   | East Basin        |                   | North Basin       |                   |
|----------------------|-----------------|---------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
|                      |                 | 1977 <sup>1</sup>   | 1972 <sup>2</sup> | 1977 <sup>3</sup> | 1972 <sup>4</sup> | 1977 <sup>5</sup> | 1972 <sup>6</sup> |
| UCL                  | +20             | 49.95               | 50.62             | 49.90             | 50.62             | 49.77             | 50.48             |
|                      | +15             | 49.95               | 50.52             | 49.90             | 50.60             | 49.79             | 50.68             |
|                      | +10             | 49.91               | 50.76             | 49.92             | 50.64             | 49.79             | 51.26             |
|                      | +5              | 49.97               | 51.02             | 49.90             | 51.30             | 49.79             | 51.24             |
|                      | +2              | 50.93               | 51.52             | 50.83             | 51.78             | 50.37             | 51.64             |
| HTZ                  | -2              | 61.60               | 60.08             | 61.45             | 59.82             | 60.56             | 58.44             |
|                      | -5              | 61.60               | 59.98             | 61.43             | 59.82             | 60.54             | 58.44             |
| LCL                  | -10             | 61.54               | 59.86             | 61.41             | 59.84             | 60.02             | 58.50             |
|                      | -15             | 61.54               | 59.86             | 61.47             | 59.84             | 59.98             | 58.26             |
|                      | -20             | 61.52               | 59.74             | 61.47             | 59.76             | 59.51             | 57.30             |
|                      | -30             | 61.52               | 59.84             | 61.45             | 59.74             | 59.31             | 56.90             |
|                      | -100            | 61.52               | 59.80             | —                 | —                 | 55.0+             | 55.60             |
|                      | -150            | —                   | —                 | —                 | —                 | —                 | 53.72             |
| Mean LCL<br>value    |                 | 61.53               | 59.82             | 61.45             | 59.79             | —                 | —                 |

Depth is given relative to this interface (see Fig. 2). In the upper line of the HTZ lines the maximum temperatures are indicated. (Bathy-sonde values corrected after reversing thermometer readings.)

<sup>1</sup> Station SO 2-160 BS+H: 21°20.68' N 38°04.92'

<sup>2</sup> Station VA3-362 BS+H: 21°20.14' N 38°05.01'

<sup>3</sup> Station SO 2-140 BS+H: 21°21.63' N 38°06.10'

<sup>4</sup> Station VA3-372 BS+H: 21°25.51' N 38°05.24'

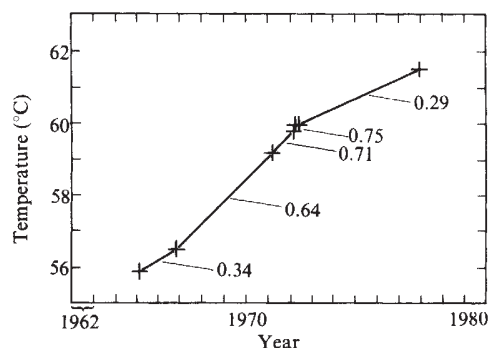
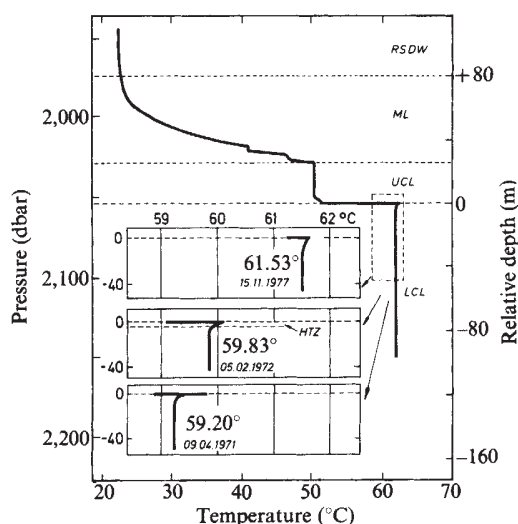
<sup>5</sup> Station SO 2-178 BS+H: 21°25.81' N 38°04.44'

<sup>6</sup> Station VA 3-346 BS+H: 21°25.79' N 38°04.66'

rate and the lack of the high temperature zone as evidence of a considerable change in the hydrothermal activity.

There are two possible causes: first, the intensity of discharge has slowed down or second, the temperature of the inflowing brine has dropped considerably compared to 1971 and 1972. It is, at present, not possible to decide between these possibilities.

**Fig. 2** Temperature–depth profile of the Atlantis II Deep brines which was recorded in November 1977 at station SO2-160 BS+H. The insets give details of the high temperature zone just below the interface of the upper convection layer (UCL) and the lower convection layer (LCL). The temperature peaks are caused by accumulating hot brine which rise as plumes due to their slightly lower density. The fading of these temperature peaks with time is the best indication of the decrease of the hydrothermal activity.



**Fig. 3** Mean heating rates of the Atlantis II Deep lower convection layer. The heating rates culminated in 1971–72 and slowed down during the past 5 yr.

As shown in Fig. 3, the period since the discovery of the Atlantis II Deep in 1965 represents a nearly complete cycle of increasing and decreasing hydrothermal activity. According to earlier results<sup>10</sup> this cycle is accompanied by changing conditions within the brine and with changes in the amount and composition of the material precipitating from the brines. Taking as a rough estimate a sedimentation rate of 1 m kyr<sup>-1</sup> (ref. 11), during the last cycle, a sediment layer of more than 10 mm should have formed since 1965. Due to the very soft or nearly fluid sediment, this uppermost layer cannot be sampled. Sediment cores from the Atlantis II Deep, however, show that this scale of microlayering (mm to cm) which represents the continuously changing supply of metal sulphides, iron-hydroxides and silicates, is characteristic for the younger sediment sections of the Atlantis II Deep.

Frequently changing hydrothermal activities are characteristic of continental geothermal areas. The activity of geysers, for example, depends on surface parameters such as rainfall, morphology, and sub-bottom drainage<sup>11,12</sup>. The triggering mechanism for pulsation in the Atlantis II Deep is not known; it may be due to tectonic movements along the steep faults of the basin, or to the self sealing of discharge vents. Examples of anhydrite coatings in vents from the floor of the South-west Basin have already been described<sup>3</sup>.

The investigations were carried out on board of RV *Sonne*. The cooperation with the PREUSSAG staff, especially Drs H. Bäcker and J. Lange, is acknowledged. Financial support of the Deutsche Forschungsgemeinschaft is acknowledged. We thank the Saudi Sudanese Commission for the Exploitation of the Red Sea Resources for permission for these investigations.

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## Lower Tertiary laterite on the Iceland–Faeroe Ridge and the Thulean land bridge

CORES of a lower Tertiary lateritic palaeosol resting on basalt were recovered<sup>1</sup> from Deep Sea Drilling Project Site 336 (Leg 38) on the north-east flank of the Iceland–Faeroe Ridge (Fig. 1), a major aseismic oceanic ridge that, together with Iceland, forms the Icelandic transverse ridge<sup>2</sup>. The transverse ridge extends from the West European continental margin to the East Greenland continental margin, forming the geographic boundary and a partial barrier to flow of water between the Norwegian–Greenland Sea to the north and the northern North Atlantic Ocean to the south. The palaeosol indicates that at least part of the Iceland–Faeroe Ridge was above sea level during early Tertiary time<sup>3</sup>. Palaeogeographic and palaeoceanographic reconstructions suggest that it formed the main part of the Thulean land bridge that connected South-east Greenland and the Faeroe islands during the early Tertiary<sup>4</sup>. This report summarises the subsidence history of the Iceland–Faeroe Ridge relative to early Tertiary seafloor spreading, basaltic volcanism, and the development of the proposed Thulean land bridge.

In the early Tertiary, the Norwegian–Greenland Sea, northern North Atlantic Ocean, and Labrador Sea–Baffin Bay areas were characterised by seafloor spreading and extensive basaltic volcanism along spreading ridges, on intraoceanic volcanic plateaus, and over trailing continental margins. Seafloor spreading occurred in the Norwegian–Greenland Sea along the then-continuous Knipovich–Mohs–Aegir Ridge<sup>5,6</sup>, in the North Atlantic along the Reykjanes Ridge<sup>7,8</sup>, and in the Labrador Sea–Baffin Bay area along Ran Ridge and the extinct Labrador Sea ridge<sup>9,10</sup>. Typical oceanic basalts of early Tertiary age were produced by seafloor spreading along each of these spreading ridges<sup>11</sup>.

Extensive continental-margin basaltic volcanics of early Tertiary age were also produced on the Vöring Plateau<sup>12</sup>, Faeroe Islands<sup>13</sup>, northwestern Britain<sup>14</sup>, Northern Ireland<sup>15</sup>, Rockall Plateau<sup>16</sup>, East Greenland<sup>17</sup>, West Greenland<sup>18</sup> and Baffin Island<sup>19</sup>. Radiometric ages for these basalts range from ~60 to 45 Myr, or late Palaeocene to middle Eocene; associated felsic plutonic rocks in some areas have slightly younger ages, probably because the more deeply buried plutonic rocks cooled more slowly. These widespread basalts record the initiation and continuation of seafloor spreading, the drift of the North American, European and Greenland plates, and the probable activity of mantle plumes. Geochemistry of the lower Tertiary basalts of the Faeroe Islands<sup>20</sup> and the upper Tertiary basalts of Iceland<sup>21</sup> suggests derivation from mantle plumes.

A thickness of approximately 30 m of basalt was recovered from the ridge at Site 336 (ref. 4), starting at a depth of 484.5 m below the sea floor, which is situated 811 m below sea level at the site<sup>1</sup>. The basalt yields K/Ar ages of  $40.4 \pm 3.2$  and  $43.4 \pm 3.3$  Myr, middle or late Eocene<sup>12</sup>. Schilling<sup>22</sup>, on the basis of geochemical analyses of the least-weathered samples, concluded that the basalts at Site 336 had a mantle-plume origin.

Because early Tertiary vertebrates in western North America, northeastern North America, and northern Europe are strikingly similar, vertebrate palaeontologists have proposed that two transatlantic land bridges provided connections between Europe and North America<sup>23</sup>. The proposed southern route extended from the Faeroe Islands to South-east Greenland and is known as the Thulean route; the proposed northern route extended from northern Norway to Svalbard and thence to northern Greenland and is known as the DeGeer route. McKenna<sup>24</sup> indicated a close similarity between land mammals in Europe and North America into the early Eocene, but major differences since the middle Eocene.

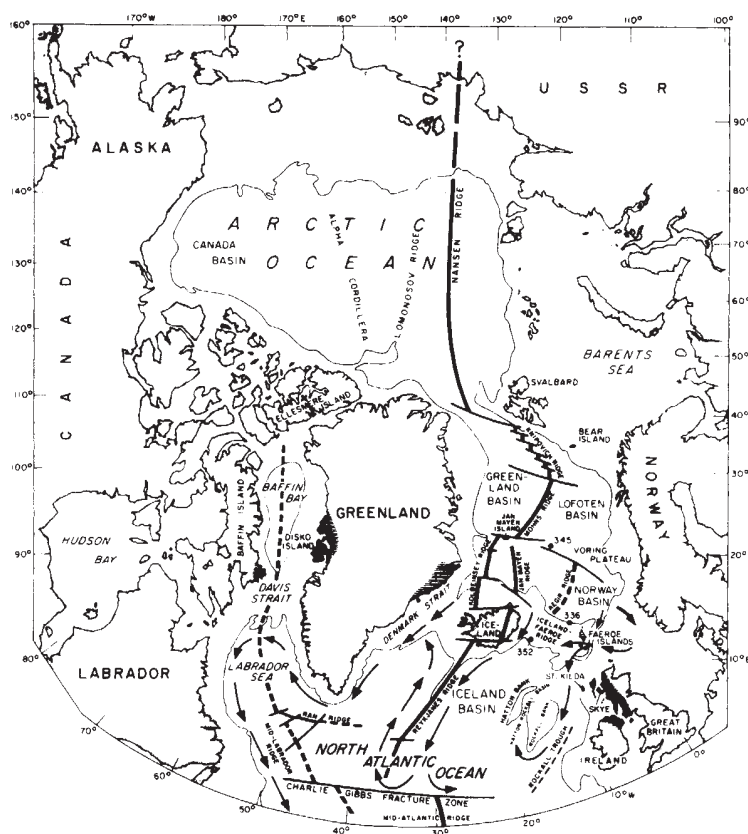
Plate-tectonic reconstructions of the northern Norwegian–Greenland Sea by Pitman and Talwani<sup>5</sup> indicated that Greenland and Svalbard were quite close, if not joined, until 47 Myr ago, or early Eocene time; termination of the DeGeer route, then, would coincide with the apparent early Eocene development of different vertebrate faunas. Talwani and Eldholm<sup>6</sup> subsequently revised the estimate of Greenland–Svalbard separation to 38 Myr ago or late Eocene on the basis of newer interpretations of magnetic anomalies, thus clouding the previous temporal correlation with vertebrate differentiation. At 38 Myr ago, waters of the Arctic Ocean were able to flow southwards into the Norwegian–Greenland Sea, but may not have been able to reach the North Atlantic because the Iceland–Faeroe Ridge formed a transverse obstruction. However, the subsidence history of the Iceland–Faeroe Ridge, the presumed Thulean land bridge, is equally unclear and permits widely different palaeogeographic scenarios.

If one accepts Schilling's<sup>22</sup> conclusion that basalts of the Iceland–Faeroe Ridge are of a mantle-plume derivation, then the ridge probably developed as a large plateau in the early Tertiary by extrusion of subaerial and submarine basalts astride the then-active Aegir and Reykjanes Ridges, possibly adjacent to one or more transform faults, as suggested by Talwani and Eldholm<sup>6</sup>. About 25–30 Myr ago, the Aegir Ridge became inactive, and spreading in the Norway Basin jumped westwards to the extinct Iceland Plateau ridge of Talwani and Eldholm<sup>6</sup>, located west of Jan Mayen Ridge, a continental fragment, and east of the now active Kolbeinsey Ridge<sup>5,6</sup>. At the same time, the proposed mantle plume beneath the Iceland–Faeroe Ridge may have shifted westwards to a point beneath Iceland, or, more probably, the Iceland–Faeroe Ridge mantle plume remained in a fixed position and the lithosphere shifted eastwards relative to it. During the past 30 Myr, Iceland has developed as a large subaerial and submarine basaltic plateau<sup>11</sup>, although the oldest exposed rocks on Iceland have yielded K–Ar dates of about 16 Myr (ref. 25).

The flat-topped crestal part of the Iceland–Faeroe Ridge is at present about 400 m below sea level; acoustic basement, which is probably basalt, crops out over many parts of it<sup>2,26</sup>. The basalt recovered at Site 336 is approximately 900 m below the top of the ridge, suggesting that at the time the area of Site 336 subsided below sea level, the Iceland–Faeroe Ridge may have been as much as 900 m above sea level. As basalts from the crest of the ridge have not been dated and basalts were not penetrated by drilling at Site 352 on the south-west flank of the ridge (Fig. 1), it is not known if the topographically higher basalts on the crest of the Iceland–Faeroe Ridge are younger than those from the flank at Site 336.

The stratigraphy, clay mineralogy, major element and trace element geochemistry, sedimentary structures, and subaerial characteristics of the 30-m-thick laterite or lateritic palaeosol resting on basalt at Site 336 are described in detail by Nilsen and Kerr<sup>3,4</sup>. The lateritic unit is overlain by almost 300 m of microfossil-bearing marine mudstone, sandy mudstone, and claystone of middle Eocene to late Oligocene age<sup>1,4</sup>. This unit was deposited at outer neritic to upper bathyal depths (120–600 m) and is overlain unconformably by 170 m of marine and glaciomarine Pliocene(?) and Pleistocene clay, mud and sandy mud. No sediments of latest Oligocene to at least early Pliocene age were recovered at either Site 336 or Site 352 (ref. 1). Various combinations of subsidence, erosion, and nondeposition can explain this unconformity. Initiation of relatively rapid flow of water over the crest of the ridge when it submerged may have prevented much sedimentation on both the ridge crest and adjacent flanks; southwards movement over the crest of colder Norwegian Sea overflow water inhibits sedimentation on the ridge crest at present<sup>27</sup>.

The subsidence history of the Iceland–Faeroe Ridge can be partly determined by palaeobathymetric interpretations of the fossiliferous marine sequence overlying the lateritic palaeosol. Accepting 45 Myr as an estimate of the oldest possible age of the basalt and 22 Myr as the youngest possible age of the



**Fig. 1** Index map of the Norwegian-Greenland Sea, Arctic Ocean, North Atlantic Ocean, and adjacent areas, showing location of DSDP Site 336 (modified from ref. 11). Light-dashed line represents 1,000-m depth contour; heavy lines, spreading ridges, dashed where extinct; light lines, transform faults; darkened areas, outcrops of basalt of the early Tertiary North Atlantic igneous province; numbered dots, selected DSDP sites; arrows indicate bottom flow of Norwegian-Greenland Sea overflow water (modified from ref. 27).

Oligocene marine sediments, then the lateritic palaeosol from time of formation could have subsided as much as 600 m in 23 Myr, a subsidence rate of  $26.1 \text{ m Myr}^{-1}$ . This average subsidence rate can be compared with that of spreading ridges and other aseismic ridges. The elevation of normal ocean crust generally decreases from about  $2,500 \pm 300 \text{ m}$  at active spreading ridges to  $5,800 \pm 300 \text{ m}$  for early Cretaceous to late Jurassic crust<sup>28</sup>. The subsidence of normal ocean crust takes place more rapidly at first, and then gradually slows through time; for the first 70 Myr, the depth and the square root of the age of the ocean floor are linearly related<sup>29</sup>. Based on simple calculations from plots of age of crust against depth for the North Atlantic ocean (Fig. 13b in ref. 28), crust 0–20 Myr old subsides at an average rate of  $80 \text{ m Myr}^{-1}$ , crust 20–40 Myr old at  $33 \text{ m Myr}^{-1}$ , and crust 40–60 Myr at  $23 \text{ m Myr}^{-1}$  (Fig. 2).

Most aseismic ridges, whose crests form near or above sea level, subside at rates comparable to that of normal oceanic crust<sup>30</sup>. Based on simple calculations from plots of Myr after formation of the aseismic ridge against depth for five aseismic ridges in the Pacific, Atlantic and Indian Oceans (ref. 30, Fig. 4), aseismic ridge crests subside at average rates of  $67 \text{ m Myr}^{-1}$  for the first 20 Myr,  $33 \text{ m Myr}^{-1}$  for the second 20 Myr, and  $20 \text{ m Myr}^{-1}$  for the third 20-Myr interval.

The estimated rate of  $26.1 \text{ m Myr}^{-1}$  from 45 to 22 Myr ago for the Iceland-Faeroe Ridge, whose underlying oceanic crust is probably 60–30 Myr old, is thus less than the average rates for ridge-generated oceanic crust and other aseismic ridges. This conclusion had been reached by other workers<sup>30,31</sup> and has profound implications regarding the history of the Thulean land bridge.

To reach its present elevation of 1,300 m below sea level, a continued uninterrupted subsidence rate from 22 Myr ago to the present of  $31.8 \text{ m Myr}^{-1}$  is required for the lateritic palaeosol. This subsidence rate is based on the assumptions that there have been no permanent major changes in sea level since 45 Myr ago, and that nondeposition rather than erosion and no uplift of the ridge from 22 to 3 Myr ago occurred. The

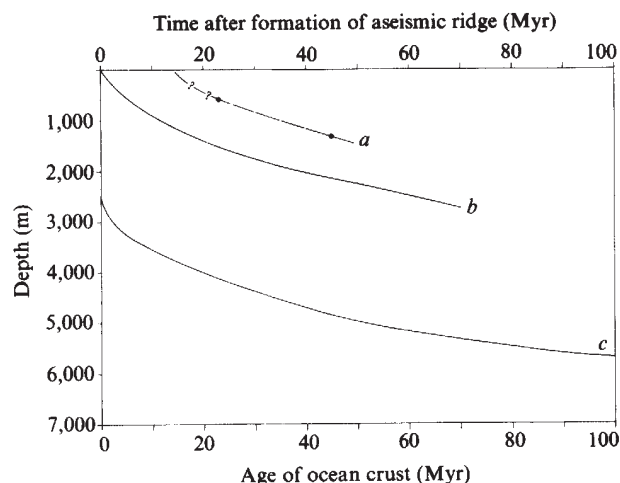
$31.8 \text{ m Myr}^{-1}$  subsidence rate is intermediate between the average subsidence rates of  $40 \text{ m Myr}^{-1}$  for 20–40 and  $40\text{--}60 \text{ Myr}$  old ridge-generated oceanic crust, respectively. It is also about equal to the  $30 \text{ m Myr}^{-1}$  average subsidence rate for an aseismic ridge 25–45 Myr after its formation. Thus, the Miocene to Holocene history of subsidence of the Iceland-Faeroe Ridge seems to be similar to that of normal oceanic crust and other aseismic ridges. This rate probably reflects cessation of mantle-plume activity beneath the Iceland-Faeroe Ridge and basaltic volcanism on the ridge in the Neogene. The pre-Miocene anomalously slow rate of subsidence may thus be related to continued basaltic volcanism and the tendency for crustal uplift associated with the underlying mantle plume.

Assuming an average subsidence rate of  $28.9 \text{ m Myr}^{-1}$  for the Tertiary, it would have taken 31 Myr for the top of the Iceland-Faeroe Ridge to subside to sea level if no additional sediments or basalts accumulated on the ridge crest after 45 Myr. The ridge crest, then, would have sunk below sea level about 14 Myr ago, during Miocene time. A sediment-filled trough along the southeastern edge of the ridge contains 100–200 m of undated sediment; using the same average subsidence rate of  $28.9 \text{ m Myr}^{-1}$ , the lowest part of the trough would have subsided below sea level about 20–24 Myr ago, during late Oligocene to early Miocene time. If the Iceland-Faeroe Ridge formed the Thulean land bridge, it should not have been interrupted or broken before this time.

Sediment ridges in the northern North Atlantic, such as Gardar on the east flank of Reykjanes Ridge and Feni in Rockall Trough, deposited by overflow of Norwegian Sea water, have developed during the past 27 Myr (ref. 32). These ridges may thus record the initiation of flow of northern water over the crest of the Iceland-Faeroe Ridge. Other workers<sup>31</sup> suggested that the Iceland-Faeroe Ridge subsided below sea level in middle to late Oligocene time, and that by 21 Myr the present general circulation pattern of the North Atlantic had become established. Faunal differences between DSDP Sites 336 and 352 support this hypothesis. It is also possible,



however, that the sediment ridges could have formed by flow of Norwegian Sea water eastwards around the Faeroe Islands through the Faeroe–Shetland Channel, thus bypassing the top of the Iceland–Faeroe Ridge. Sclater *et al.*<sup>31</sup> suggested that the Iceland–Faeroe Ridge began to subside after the spreading ridge beneath it jumped westwards about 25–30 Myr; however, the laterite and overlying Eocene to upper Oligocene marine sediments clearly indicate that the ridge was subsiding before this.



**Fig. 2** Subsidence curves for *a*, the Iceland–Faeroe Ridge (DSDP Site 336); *b*, normal aseismic ridges (from ref. 30, Fig. 4); and *c*, North Atlantic normal ocean crust (from ref. 29, Fig. 13b).

The range of possibilities regarding the subsidence history of the Iceland–Faeroe Ridge and the extent of the Thulean land bridge is still great. My calculations suggest that the lowest part of the ridge crest subsided below sea level 20–24 Myr ago. North American and European vertebrate remains indicate that the land bridge was disrupted in early or middle Eocene time (46–51 Myr ago), before the age of formation of the basalt penetrated at Site 336. It must be concluded from available data that the Iceland–Faeroe Ridge probably did not extend as a subaerial ridge completely across the widening North Atlantic and Norwegian–Greenland Sea during late Eocene and Oligocene time but was broken or interrupted, possibly along its western connection with Greenland. However, because the DeGeer route across Svalbard was apparently not broken until 38 Myr (ref. 6), the differences in vertebrates beginning in the middle Eocene is doubly puzzling.

The detailed history of the Thulean land bridge and the significance of the Iceland–Faeroe Ridge, despite much new data from DSDP Holes 336 and 352, is not yet fully revealed. Additional information and data required to resolve the history of the Iceland–Faeroe Ridge and the Thulean land bridge include (1) dating and analysis of basalts and sediments on the top of the ridge, (2) dating and analysis of sediments in the trough at the southeastern margin of the ridge and in the Faeroe–Shetland Channel, (3) dating and analysis of more sediments on the margin of the ridge to obtain a more complete record of sedimentation for late Oligocene and Miocene time, (4) a careful analysis of the timing and geometry of the separation of Svalbard and North-east Greenland and breaking of the DeGeer route, (5) a reevaluation of the land vertebrate data, and (6) better understanding of the Tertiary palaeogeography of Greenland itself. The last requirement raises the possibility that although the Thulean land bridge may have extended across the widening marine area, vertebrates were not able to travel from South-east Greenland to North America because of breaks within the Greenland landmass itself or between Greenland and North America. The lateritic palaeosol and

underlying basalt indicate that during the early Tertiary, warm, humid climates and mantle-plume-derived volcanic activity prevailed throughout the Norwegian–Greenland Sea area. This volcanic activity may have produced the Thulean land bridge between Greenland and northwestern Europe during the early Tertiary. The Iceland–Faeroe Ridge would have exerted a controlling influence over oceanic circulation, especially on southwards flow of Norwegian Sea waters, as inferred by Vogt<sup>33</sup> before the drilling at Site 336, and thus a strong controlling influence on sedimentation in the adjacent North Atlantic Ocean and Norwegian–Greenland Sea<sup>34</sup>.

I thank Dennis R. Kerr for analysing samples of the lateritic palaeosol, Wendel Duffield and Richard Poore for helpful reviews of this manuscript, and my shipboard colleagues on Leg 38 for stimulating discussions.

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## Anomalous upper mantle structure beneath the Cretaceous Fogo seamounts indicated by P-wave reflection delays

TRAVEL-TIME residuals of teleseismic PP waves, reflected approximately halfway between the source and receiver, may give information on seismic velocity near the reflection point. Effects due to structure near the source and receiver are minimised by subtracting the residual of the direct P-wave from that of the PP arrival<sup>1</sup>. If these differential PP–P residuals are available for closely spaced reflection points, a contour map of compressional wave delays for the reflecting region can be obtained, which may then be related to crustal and upper mantle structure. The contours of two-way delay times for

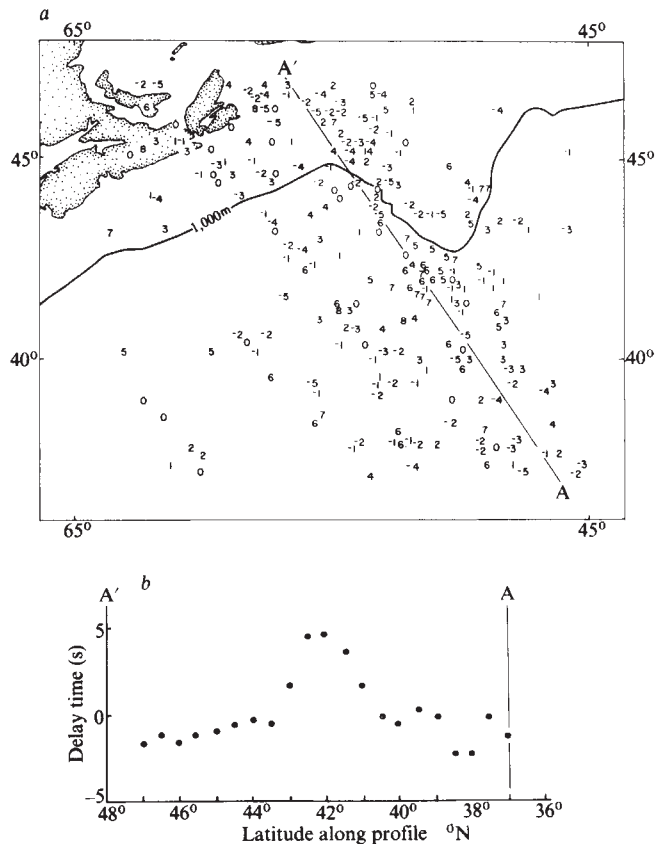
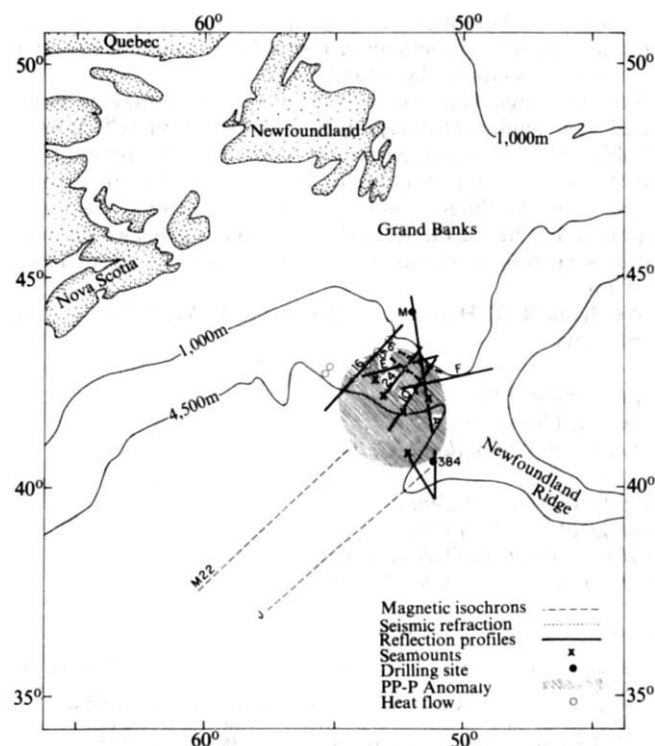


P-waves reflected beneath Atlantic Canada have been presented<sup>1</sup>, and show an area of late reflections south of the Grand Banks, centred at about 42°N, 52°W. This area coincides with the Fogo seamounts (Fig. 1) and the relationship between these near-surface features and the P delays is examined here in more detail.

The Fogo seamounts are volcanic structures which rise 2–5 km above the level of the surrounding volcanic oceanic crust. They lie immediately south of the Grand Banks and are on line with the Newfoundland Ridge<sup>2</sup>. The locations of the seamounts and their heights above the surrounding basement were obtained from the seismic reflection lines shown in Fig. 1, using sediment velocities reported by Jackson *et al.*<sup>2</sup>. The reflection lines show that the seamounts are partially or totally buried beneath sediments now forming the lower continental slope<sup>3,4</sup>. No seamounts have been located west of line 16 (Fig. 1)<sup>5,6</sup>. Basaltic and trachytic rocks of early Cretaceous age have been recovered in the Mallard exploratory well on the southwestern Grand Banks (Fig. 1) and this volcanism may have been associated with the formation of the Fogo seamounts. The seamounts are separated from the Newfoundland Ridge by the northeasterly trending Spur Ridge which is underlain by a basement high and is associated with the high amplitude *J* magnetic anomaly<sup>4,7</sup>. The entire region, comprising the southern margin of the Grand Banks and the Newfoundland Ridge, is thought to be a fracture zone, active in the early stages of the plate separation of Africa and North America<sup>8–10</sup>.

The age of the sea floor near the Fogo seamounts is shown from magnetic anomaly studies to be early Cretaceous<sup>11</sup>. This is supported by DSDP drilling results at Site 384 (Fig. 1) on the Spur Ridge where oceanic basement was dated at about 115 Myr BP (ref. 12). One of the most interesting drilling results at this site is that the Spur Ridge has subsided dramatically, about 4,100 m, since the late early-Cretaceous when it was close to sea level. The *J* anomaly and associated basement structures define isochrons on both sides of the Atlantic and

**Fig. 1** Location of the Fogo seamounts and the PP-P anomaly. The shaded area shows the location of PP-P values of greater than 2 s delay taken from the 5° trend surface fitted to the data. Seismic reflection and refraction lines<sup>2</sup> and heat flow stations<sup>30</sup> in the area are shown as well as drilling sites. M22 and *J* are magnetic anomaly isochrons<sup>11</sup>. *M* is the location of the Mallard well, 384 = DSDP site 384 on the Spur Ridge.



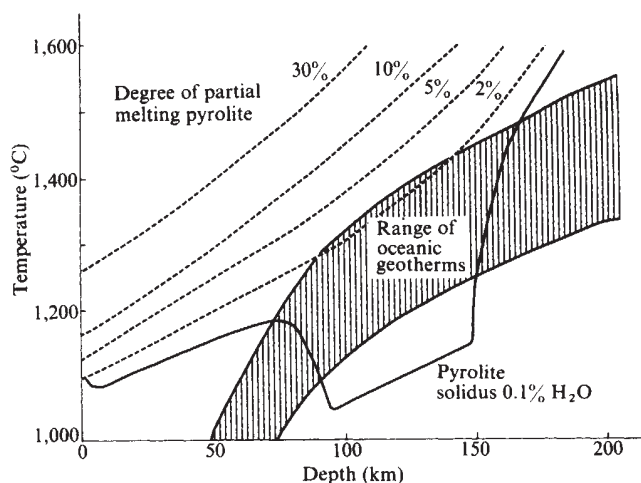
**Fig. 2** *a*, Values of PP-P delay times plotted at the PP reflection points and the location of profile AA'. *b*, Values of averaged PP-P delay times along profile AA', calculated as described in the text.

must represent a major event in the seafloor spreading history of the central Atlantic Ocean.

The Fogo seamounts are therefore apparently one element in a complex volcanic province extending eastwards from the seamounts across the Spur Ridge and along the Newfoundland Ridge. There is no direct evidence that these elements are genetically related although several previous studies have suggested that a hot spot was active there ~120 Myr BP (refs. 13, 14). If this hypothesis is correct, the volcanic province now observed might be the remaining trace of that hot spot.

The zone in which P-wave delays coincide with the Fogo seamounts (Fig. 1) is defined by about 18 late reflections, some exceeding 6 s delay<sup>1</sup>. These data are shown in Fig. 2, where values of PP-P delay times are plotted at the reflection points. The errors in reflection times and positions of reflection points have been discussed by Stewart<sup>1</sup>. These P-wave delays are consistently larger than those outside the anomalous zone. Trend surfaces of degree 5, fitted to the data over an area of 10° latitude by 15° longitude<sup>1</sup> show the same anomalous P-delay zone, somewhat reduced in amplitude, and support the contention that this zone is not an artefact caused by random scatter in data errors. The anomaly is also displayed as an average along profile AA' (Fig. 2) which is approximately normal to the great circle traces of the rays. Data within one geocentric degree were projected perpendicularly on to the line, and a running mean is taken within a window corresponding to one degree of latitude along the profile (about 150 km). The width of the anomaly is of the order of 250 km or less, and seems to be fairly sharply defined with a maximum amplitude of 5 s.

It is unlikely that these P-delays can be accounted for in terms of regional variations of crustal structure or large thicknesses of low velocity sediments within the anomalous zone. Seismic reflection and refraction measurements over the



**Fig. 3** Melting curves as a function of temperature and depth for pyrolite with 0.1% water. The range for normal oceanic geotherms<sup>19</sup> is also shown.

Fogo seamounts<sup>2</sup> (line 5, Fig. 1) and over oceanic crust outside this anomalous area<sup>15</sup> show that two-way vertical reflection times are slightly less in the crust beneath line 5 than beneath the lower continental rise off Nova Scotia. Low velocity sediments are not exceptionally thick in this area<sup>5,16</sup>. Therefore the P-wave anomaly is interpreted as being due to the presence of a plug-shaped low velocity region in the upper mantle, probably extending down to the low velocity layer. The seamounts may be the surface expression of conduits to the surface from this low velocity region.

The reflection points lie in the 40–45° distance range from the sources, giving angles of incidence below the crust of about 35° (ref. 17). Compared with a normal upper mantle P-wave velocity of about 8.0 km s<sup>-1</sup>, the plug velocity must be reduced by at least 20% over a 100 km depth range to give about 6 s delay. As this is unlikely with any reasonable compositional change, a variation of the elastic moduli with temperature must be sought.

The seismic results might be explained if a mantle blob (hot spot?)<sup>18</sup> was active in the area during the early Cretaceous. Material from the low velocity layer or even deeper may have ascended and produced high temperatures at relatively shallow depths. In Fig. 3 typical oceanic isotherms are presented on the phase diagram for a pyrolite mantle<sup>19</sup>. It shows that the low velocity layer occurs at 100 to 150 km depth where partial melting greater than 1% occurs<sup>19–21</sup>. Within the blob, the high temperature at lower pressure (depths) may have resulted in partial melting exceeding 20%. If the present day temperatures within the blob are sufficiently high to allow over 10% partial melting, the shear modulus will be reduced to about one-tenth or less of its value in a solid. This would give the required decrease in compressional velocity<sup>22</sup>.

But is it reasonable to expect a large residual effect on compressional velocity at present, in view of the fact that the blob is now inactive and the area has presumably been cooling since early late-Cretaceous time (~100 Myr BP)? The thermal history in areas of mantle upwelling (hot spots?) is not well understood<sup>23–26</sup>. Nor is it known whether the mantle blob postulated here was created in a mid-plate region, over an active mid-ocean ridge, or by local diapirism of mantle material into the fracture zone thought to lie beneath the southern margin of the Grand Banks<sup>26</sup>. The following analysis is therefore a gross approximation of the real situation. If we assume that the initial (100 Myr BP) temperature in the blob was about 1,600 °C, corresponding to about 30% partial melting<sup>19,22</sup> and that the surrounding mantle material was within the range of normal oceanic mantle temperatures (Fig. 3), then the temperature excess in the blob would have been about 1,000 °C at 20 km depth and about 500 °C at 70 km. Assuming

that the blob has 100 km radius, a thermal diffusivity of 10<sup>-6</sup> SI units, and was cut off from the source of hot mantle material and began to cool at 100 Myr BP (ref. 28) the temperature excess in the centre of the plug would have dropped at the present time to about half its initial value. A present temperature excess of 500–200 °C in the upper mantle would allow partial melting of about 10%, consistent with the observed P delay anomaly.

This explanation of the observed data should be verifiable by heat flow measurements within the area occupied by the mantle plug and by an examination of long wavelength gravity anomalies. The presence of the continent–ocean transition near the anomalous P delay zone and the perturbation of the free-air gravity anomalies by the seamount topography complicate the interpretation of the gravity field. Free-air gravity, averaged in 5° by 5° areas, shows a residual gravity anomaly of 20 mgal centred on the Grand Banks and extending south across the continental margin into the anomalous zone<sup>29</sup>. Positive residual anomalies of 10–20 mgal are associated with areas known to be affected by excessive volcanism such as Iceland and the Azores and with old volcanic areas such as Bermuda. However, the location of the gravity anomaly centre somewhat north of the low velocity mantle zone proposed here suggests that the anomaly may be caused primarily by continental structures underlying the Grand Banks.

Six heat flow measurements within approximately 200 km of the centre of the anomaly (Fig. 1) have a mean value of 44 (±4) mW m<sup>-2</sup> (ref. 30). Of the three measurements on the edge of the anomaly, one is significantly higher, 59 (±7) mW m<sup>-2</sup>, than the mean and the elevated temperature gradient at this point is consistent with this interpretation of the reflection delays. However, the three heat flow measurements are not all high; measurements near the centre of the anomaly are needed.

If the anomalous low-velocity zone is interpreted as indicating the presence of a hot spot or blob in the area during the early Cretaceous, the subsidence of the region might be larger than for normal oceanic crust of the same age because of the greater initial updoming of the asthenosphere by the blob. The large subsidence of the Spur Ridge<sup>12</sup> would support this reasoning; similar effects have been observed for oceanic island chains in the Pacific<sup>25</sup>. However, this P-wave and seamount anomaly is not lineated but localised, suggesting that a mantle plume mechanism was not operating continuously over an extended period of time or that the anomalous mantle zone was formed instead by dispersion of low-velocity material into the fracture zone south of the Grand Banks.

Note that regions of past volcanism elsewhere may be associated with significant delays in P-wave travel times<sup>31</sup>. Where suitable source–receiver geometries exist, the delay times of PP waves may be useful in defining structures such as that associated with the Fogo seamounts. Only published data are required, and hence the analysis can cover regions which may not be accessible using other methods, without an appreciable field effort.

We thank R. T. Haworth, M. Keen and J. Wright for helpful discussions.

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Received 15 May; accepted 22 June 1978.

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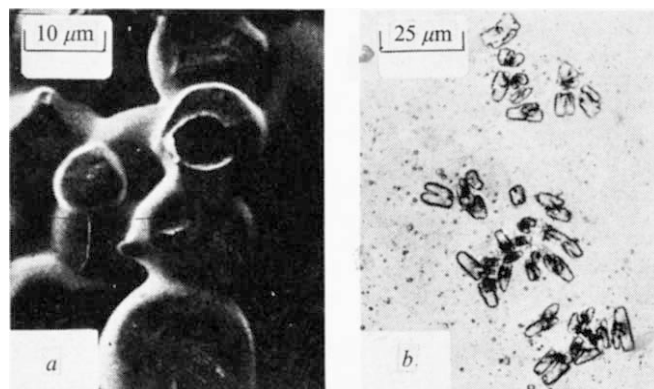
## A possible mechanism of ice splinter production during riming

IN supercooled clouds of particular characteristics—notably a broad droplet size distribution—the concentrations of ice particles at a given level may exceed by several orders of magnitude the measured concentrations of ice nuclei.<sup>1-3</sup> These observations have important implications with respect to the efficacy of techniques for the artificial enhancement of rainfall. There is good evidence<sup>4,5</sup> supported by calculations<sup>6,7</sup> that the secondary ice particles form during the process of riming. However, the physical mechanisms by which these particles are produced has not been identified. We describe here some experiments which suggest that the bursting of supercooled droplets as they freeze onto a riming ice particle may be responsible. This fragmentation process is well established for isolated drops in air, which may lose heat more-or-less symmetrically to their environment as they freeze, thus permitting the formation of a closed shell of ice within which immense pressures may develop as freezing proceeds. However, no generally accepted evidence exists for the occurrence of this process during the growth of rime.

In experiments into the initiatory stages of the riming process, frost crystals of typical dimensions 100  $\mu\text{m}$  were gently detached from the paper surface on which they had grown, inside a cold room, and fell a distance of about 5 cm before entering a chamber filled with supercooled cloud. By this time they were in thermal equilibrium with their environment and were within 10% of their terminal fall speeds. The cloud had been formed by passing steam into the chamber, which was of depth 2 m, and allowing it to stabilise for  $\sim 40$  min. Tests showed that beyond this stage the liquid water content and droplet size distribution remained effectively constant for the period of the experiments. Typically the size distribution (measured by sedimenting droplets on to a coated slide and also using a cascade impactor) peaked at a diameter around 8  $\mu\text{m}$ , with 99% of the droplets being smaller than 20  $\mu\text{m}$ , and the liquid water content (measured using an impaction technique) lay between 7 and 14  $\text{g m}^{-3}$ . After they had fallen through the cloud some of the crystals were collected on a glass slide coated with thermalised formvar solution. The plastic replicas of the rimed ice crystals so obtained were subsequently coated with a film of gold and examined by an electron scanning microscope (ESM), which produced high-magnification photographs of the interesting features.

Figure 1a is an ESM photograph of a number of droplets frozen on to the surface of an ice crystal which had swept them up; the jagged dark central holes are artefacts and should be discarded. Of particular interest is the droplet, of diameter around 15  $\mu\text{m}$ , which is in the middle of a line of three droplets and which exhibits a protuberance, about 3  $\mu\text{m}$  long. This is a clear example of a 'spike' produced when the ice shell surrounding the liquid interior of a drop freezing fairly symmetrically, is ruptured at a single weak point. Several such frozen drops with protuberances were found in these experiments, and the characteristic shapes are very similar to those frequently found on natural ice crystals, in the early stages of riming, which settle to ground from wintertime cap clouds over Wyoming (C. P. R. Saunders and G. Vali, personal communication).

If the ice shell relieves the pressure by giving way at a single weak spot, thus forming a protuberance, it is unlikely that ice fragments will be ejected. However, these replicated objects are informative in that they indicate that the heat loss from the freezing drop was sufficiently symmetrical to permit the formation of an ice shell. Earlier work has shown that the alternative mechanism of pressure-release is 'drop-shattering', in which a considerable proportion of the ice shell is ejected in the form of fragments of ice. This process could give rise to ice particle multiplication during riming. The ESM photographs display irregularly shaped droplets frozen onto the ice crystals but it is not possible to determine unequivocally whether these are a consequence of drop-shattering. However, Fig. 1b may provide direct evidence of the fragmentation process. It shows three clusters each of around 10 ice fragments collected just downwind of an ice-coated sphere exposed to a stream of supercooled water droplets in experiments<sup>8</sup> principally designed to study electrical effects during the riming process. In this particular case the air temperature was  $-7^\circ\text{C}$ , the air-stream velocity was  $2 \text{ m s}^{-1}$  and the droplets, produced by a spinning top device, possessed a range of sizes, with a mode diameter around 35  $\mu\text{m}$ . However, similar clusters were found for a wide range of values<sup>8</sup> of size, temperature and air speed. No ice fragments were found when either the target or the droplets were absent from the air stream.



**Fig. 1** a, Electron scanning microscope photograph of a part of a gold-plated replica of an ice crystal of size around 200  $\mu\text{m}$  collected after riming at  $-8^\circ\text{C}$ . b, Three clusters of ice fragments collected downwind of an ice-coated sphere riming at  $-7^\circ\text{C}$ .

These experiments, considered together, suggest that secondary ice particles may be produced during the riming process as a consequence of 'drop-shattering' on freezing. The necessary approximately-symmetrical heat loss may be achieved if the drops in the cloud possess a range of sizes and a larger drop impacts on to a smaller one already frozen onto the riming crystal. In this case the rate of flow of heat from the freezing drop in to the ice substrate is restricted, because of the limited interfacial area, and a significant loss of heat to the surrounding air can occur, thus providing the conditions under which a strong and closed ice shell, necessary for fragmentation, can form. It



has been reported<sup>4,5,9</sup> that a range of droplet sizes is necessary for ice splinter production to accompany the riming process.

These experiments are far from conclusive. In particular, they do not explain the observed sensitivity of splinter production to temperature<sup>4,5</sup>. However, they do support the idea that an established physical process—drop-shattering—is responsible for this multiplication effect. More detailed experiments are in progress which will be complemented by ones in which the fine structure of rime is studied as a function of the geometrical and thermal characteristics of the substrate on which it is formed.

This research was supported by grant GR3/3007 from NERC.

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## Clean surface reactions between diamond and steel

THE rate of wear of diamond grinding on machining steel is known to be extremely high. For example, the wear rate of a diamond turning mild steel is  $10^4$  times greater than when turning brass of comparable hardness<sup>1</sup>. Several discussions of this wear conclude that it is due principally to the high local flash temperatures generated during machining and to the catalytic properties of the steel. Static experiments show that diamond graphitises at temperatures above 1,800 K in vacuo, or above 1,100 K in the presence of iron, and other experiments that flash temperatures of this order may be generated by machining. Hence, there is now general agreement that the wear proceeds by the graphitisation of the diamond; the surface layer of graphite being continually removed either by the abrasion of the steel or by solution in the steel<sup>2-7</sup>. However, little information is available on the actual conditions prevailing at the interface during machining. Therefore, we have studied the mechanism of wear more closely by making quantitative measurements of the wear rates of round-nosed diamond tools turning billets of mild steel under well defined conditions.

At a linear cutting speed of  $11 \text{ m s}^{-1}$ , a reduction of the air pressure from 760 to 0.1 torr causes the wear rate to fall by the order of 50%. As oxygen reacts readily with diamond at elevated temperatures, this suggests that under normal cutting conditions the oxygen in the air acts as a catalyst for the graphitisation. On increasing the cutting speed, a critical value is reached, after which the wear proceeds very rapidly indeed; at  $30 \text{ m s}^{-1}$  in air it is about 50 times greater than at  $11 \text{ m s}^{-1}$ , and is accompanied by a shower of white sparks. If at this speed the air pressure is reduced to 0.1 torr, or is replaced by an atmosphere of argon, no hot debris is seen, but the wear rate remains unchanged. We therefore identify this extremely high wear as the conversion of diamond to graphite, due to high local flash temperatures at the steel–diamond interface.

Other experiments have used a cutting speed of about  $0.16 \text{ m s}^{-1}$  to effect a considerable reduction of the flash temperatures. The wear rate in 760 torr of air was usually

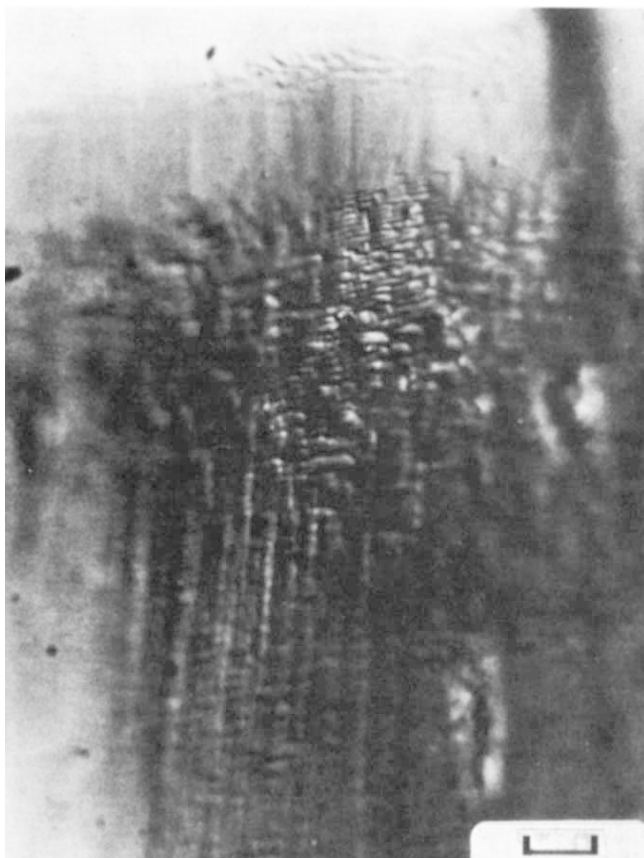


Fig. 1. Optical micrograph of the flank of a diamond tool after turning mild steel. Scale bar,  $10 \mu\text{m}$ .

substantially reduced below its value at  $11 \text{ m s}^{-1}$ , as would be expected. However, it was also found, when turning at  $0.16 \text{ m s}^{-1}$ , that reducing the air pressure from 760 to 0.1 torr had the effect of substantially increasing the wear by a factor of order 5, to a value greater than that observed in 760 torr of air at  $11 \text{ m s}^{-1}$ , a cutting speed 70 times higher.

An estimate of the flash temperatures at the interface is obtained by noting that a cutting speed of  $30 \text{ m s}^{-1}$  was sufficient to produce white hot debris, which implies that the excess flash temperatures at this speed were of the order of 1,000 K. Hence a conservative estimate of the flash temperatures at  $0.16 \text{ m s}^{-1}$  gives an upper limit of 100 K above ambient. This estimate was confirmed by subsidiary experiments with a semi-conducting diamond tool turning brass and copper billets, when the thermal e.m.f. generated at the interface fell steadily with decreasing speed. Two further experiments were made to investigate the effect of temperature on the reaction. The cutting speed in 0.1 torr was reduced from  $0.16$  to  $0.02 \text{ m s}^{-1}$ , and the wear rate actually increased. Finally, the wear rate under reduced pressure at  $0.16 \text{ m s}^{-1}$  was observed cutting first normally, and then with a billet maintained at  $220^\circ\text{C}$ . The wear rate was the same in both cases, and was unaffected by the higher temperature. Hence, the wear process under these conditions is not thermally activated.

Bulk diamond and steel do not react when placed in contact at room temperature, so the wear in the present experiments must be due to the clean metal surfaces generated during machining having an enhanced chemical activity. Therefore, if any gaseous atmosphere can penetrate to the interface, it may contaminate the surfaces and block off the reaction. In fact, when cutting at  $0.16 \text{ m s}^{-1}$  we can distinguish two ranges of atmospheric pressure each giving a wear rate independent of pressure, the lower pressures giving the higher wear. Moreover as the wear is so high, of the order of one carbon atom for every five atoms of clean metal passing over the diamond, the diamond as well as the steel surface will be quite clean.

Figure 1 shows an optical micrograph of the flank of a tool worn at the highest rate mentioned above, which we identified with the graphitisation process. The vertical grooving is obviously caused by the metal moving vertically downwards over the face of the diamond. The surface also exhibits a system of almost horizontal ridges and grooves which cannot have been produced by any form of abrasive wear, but are rather similar to the etch-like features produced during the graphitisation of a diamond surface<sup>8,9</sup>. (As the diamond tools had random crystallographic orientations, no significance can be attached to the particular orientation of these features.) All the tools used under the various conditions mentioned above show less pronounced but geometrically similar patterns with similar linear features inclined at considerable angles to the vertical. The pattern observed after turning at  $0.16 \text{ m s}^{-1}$  in 0.1 torr is particularly similar to the others. We conclude that, even at the very low cutting speeds, the diamond is wearing away by a process similar to that which occurs at the higher speeds, and which appears to be a form of graphitisation, even though the excess flash temperatures at the interface are negligibly small.

Clean surfaces have an enhanced reactivity, but in spite of the practical importance of the wear of rubbing surfaces, very little information is available on reactions between clean solids. The present machining experiments demonstrate for one particular reaction, between diamond and steel, that this enhanced activity leads to chemical interaction at much lower temperatures than is usually considered possible. Moreover, as this interaction under virtually isothermal conditions is due essentially to clean surfaces rather than to any specific properties of steel and diamond, similarly enhanced activity is to be expected when machining other materials. A full account of this work, with the techniques employed, will be published elsewhere.

We thank De Beers Industrial Diamond Division and the SRC for support for this work.

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## Heritability of external morphology in Darwin's finches

ECOLOGISTS use measurements of avian morphological characters to test and modify evolutionary theories. But virtually nothing is known about the inheritance of such characters, although the theories are usually based on genetic models. As a result, either implicitly or explicitly, avian ecologists have used the terms phenotype and genotype almost interchangeably<sup>1</sup>. At the same time, theoreticians have assumed in their calculations that heritabilities are equal to one<sup>2</sup>. This problem is particularly acute when intraspecific variation itself is the focus of attention, as it is in studies of the niche-variation hypothesis<sup>1</sup>. We show here that several ecologically significant characters have high heritabilities in Darwin's medium ground finch (*Geospiza fortis*).

We have measured and individually marked about half of the adult *G. fortis* on Daphne Major island, in the Galápagos

Archipelago<sup>3</sup>. In 1976 we marked more than 400 nestlings, of which 82 were recaptured 3 months later when virtually fully grown. By observing marked parents at the nest, we can relate their measurements to those of their subsequently caught young, and thus calculate heritabilities.

For each morphological character we first tested males, females, and offspring separately, in each of four different one-way analyses of variance (AOVs): variation among three habitats (outer slope, inner slope and plateau<sup>3</sup>), among dates of first egg (early, middle or late), among clutch sizes, and among numbers of young fledged. Seven of the 84 AOVs were significant: offspring among habitats (d.f. = 2,79; weight  $F = 4.27$ ,  $P = 0.017$ ; wing length  $F = 3.37$ ,  $P = 0.04$ ; bill depth  $F = 5.32$ ,  $P = 0.007$ ; bill width  $F = 3.95$ ,  $P = 0.023$ ), males among numbers of young fledged (d.f. = 4,68; wing length  $F = 5.28$ ,  $P = 0.0009$ ) and offspring among numbers of young fledged (d.f. = 3,78; weight  $F = 3.08$ ,  $P = 0.032$ ; wing length  $F = 2.91$ ,  $P = 0.040$ ).

**Table 1** Parametric correlations between mates as an index of assortative mating

|                              | Successful pairs | Unsuccessful pairs |
|------------------------------|------------------|--------------------|
| Sample size                  | 40               | 21                 |
| Weight                       | 0.16             | 0.16               |
| Wing length                  | -0.09            | 0.37               |
| Tarsus length                | 0.35*            | 0.10               |
| Bill length                  | 0.31*            | 0.21               |
| Bill depth                   | 0.33*            | 0.16               |
| Bill width                   | 0.42†            | 0.22               |
| Bill length at depth of 4 mm | -0.16            | 0.02               |

The characters and measurement techniques are described elsewhere<sup>3,10</sup>.

\*  $P < 0.05$ .

†  $P < 0.01$ .

The variation in young among habitats presumably reflects differences in the quantity or quality of food available, because parents do not vary concurrently. The significant AOV of males among numbers of young fledged is due to a reduced wing length in males not fledging any young. This group may contain younger inexperienced males or birds with worn feathers, in poor condition. The variation in weight and wing length of young among numbers of young fledged is the result of a negative correlation between these characters and brood size. These characters are the last to reach adult size, and in larger broods each young may receive less food, further delaying development. The correlation probably diminishes with time.

These tests do not eliminate the possibility of genotype-environment interactions or correlations, but the absence of simultaneous differences among males, females and offspring among habitats, nesting times and so on reduces the likelihood of the most obvious biases.

There are significant correlations between mates for several characters (Table 1). Correlations are smaller and not significant in pairs which did not produce at least one fledgling. There are no records of positive assortative mating for continuous characters in birds. Assortative mating in the finches is associated with increased reproductive success, but we have no simple explanation for the mechanism or meaning of this advantage. The correlations have the effects of reducing the standard error of the regressions, and of increasing the heritabilities estimated by sib-sib correlation<sup>4,5</sup>.

All characters show large heritabilities when calculated by four different methods<sup>5,6</sup> (Table 2). Maternal effects and other characteristics of local environment do not seem to exert a major influence on heritability, as all methods give similar results<sup>5</sup>. There remains the possibility that the values are inflated by genotype-environment correlations and inter-



**Table 2** Heritabilities of seven characters using four different methods

|                              | Midparent-offspring regression | Male-offspring regression | Female-offspring regression | Full-sibs correlation | Averages |
|------------------------------|--------------------------------|---------------------------|-----------------------------|-----------------------|----------|
| Sample size                  | 26                             | 41                        | 37                          | 18                    | —        |
| Weight                       | 0.84 ± 0.23                    | 0.74 ± 0.26               | 1.10 ± 0.42                 | 0.80 ± 0.38           | 0.87     |
| Wing length                  | 0.53 ± 0.37                    | 0.38 ± 0.36               | 0.97 ± 0.49                 | 0.65 ± 0.41           | 0.63     |
| Tarsus length                | 0.43 ± 0.22                    | 0.46 ± 0.31               | 0.38 ± 0.30                 | 0.27 ± 0.44           | 0.38     |
| Bill length                  | 0.62 ± 0.22                    | 0.75 ± 0.30               | 1.17 ± 0.32                 | 1.34 ± 0.25           | 0.97     |
| Bill depth                   | 0.82 ± 0.15                    | 0.94 ± 0.23               | 0.95 ± 0.26                 | 1.41 ± 0.23           | 1.03     |
| Bill width                   | 0.95 ± 0.19                    | 1.06 ± 0.27               | 1.09 ± 0.42                 | 0.99 ± 0.35           | 1.02     |
| Bill length at depth of 4 mm | 0.48 ± 0.32                    | 0.61 ± 0.26               | 0.15 ± 0.32                 | 0.78 ± 0.39           | 0.50     |
| Averages                     | 0.67                           | 0.71                      | 0.83                        | 0.89                  | 0.77     |

Figures are  $h^2 \pm \text{s.e.}$  Midparent-offspring heritability ( $h^2$ ) is the slope of the regression of each offspring value on the average value of its parents. Male-offspring and female-offspring heritabilities are twice the slope of each offspring regressed on each parent alone. Full-sibs heritability is twice the intraclass correlation coefficient among two or more sibs. Sample sizes of the regressions are the number of different offspring used, with parents reused in the few cases where more than one offspring was measured in a single family. The full-sib analysis consists of 16 families of two sibs and two families of three sibs each, for a total of 18 families and 38 sibs. The regressions provide a direct, unbiased measure of the proportion of additive genetic variance. The sib-sib correlation sets an upper limit to heritability because it includes a common environment effect which reduces intrafamilial variance, and  $\frac{1}{4}$  of the variance due to genetic dominance. Values higher than the theoretical maximum of one are the result of doubling statistics with large standard errors.

actions. It is customary to consider the special environments correlated with particular genotypes as functional parts of those genotypes<sup>5</sup>. It may prove convenient to think of assortative mating in a similar fashion. Genotype-environment interactions, on the other hand, have been the source of considerable controversy<sup>7</sup>. We have no evidence for such interactions in our data, and have taken the traditional course of assuming that they would be included in the environmental variance term<sup>5</sup>.

One approach to genotype-environment interactions and correlations in a field study is to randomise genotypes among environments. We planned to do this on Daphne Major by randomising clutches among banded pairs. But in 1977 a drought prevented the experiment, while in 1978 there were too few breeding pairs. Nevertheless, preliminary analysis of our unrandomised 1978 data seems to substantiate the findings reported here, and we hope to randomise clutches in a future season.

What is the significance of large heritabilities for characters with obvious ecological functions? Bill length, depth and width have heritabilities  $\sim 1$ , which suggests that bill shape and size should be especially responsive to natural selection. These characters also produced the highest indices of assortative mating. These findings are important because considerable interest has been devoted to exploring the functional relationships among bill morphology, feeding ecology, and species recognition in Darwin's finches<sup>1,8-10</sup>. We are also interested in demonstrating that intraspecific variation is adaptive and is maintained by natural selection. It is therefore critical to strengthen the link between observed phenotypic and assumed underlying genetic variation<sup>1</sup>. Attempts to substantiate this link by correlating electrophoretic with morphological variation have met with some success<sup>11-13</sup>, but the relationship between variability in plasma proteins and variation in other parts of the genome remains unclear<sup>14,15</sup>. Heritability, in spite of its limitations, should give us a rough idea of how a given trait will respond to selection, and can tell us if the phenotypic variation is likely to be a good indicator of underlying genetic variation. Traits with high heritabilities do not necessarily have histories of relaxed selection, as might be expected if genetic variation was 'used up' during selection, as predicted by the fundamental theorem of natural selection<sup>5</sup>. Bulmer<sup>16</sup> shows that as the number of genes controlling a character becomes large, variability is rapidly regained when selection ceases.

Recent studies of *Melospiza melodia* (personal communication from J. N. M. Smith), *Parus major*<sup>17</sup> and *Puffinus puffinus*<sup>18</sup> have shown heritabilities of 0.46-0.92 for characters similar to ours. Thus perhaps the assumptions made previously by both empiricists and theorists concerning the relationship

between phenotypic and genetic variation in birds will prove justified.

This work was supported by NRC (Canada) and the Frank M. Chapman Memorial Fund. We thank Laurene Ratcliffe, Jaimie Smith, Kurt Sittmann and Graham Bell for assistance. The Charles Darwin Research Station arranged logistic support.

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Received 6 January; accepted 26 June 1978.

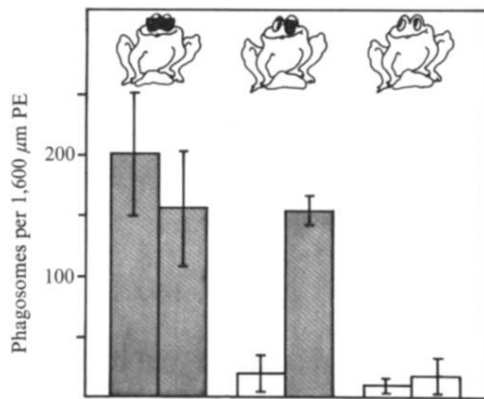
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## Photoreceptor shedding can be initiated within the eye

IN the vertebrate retina the light-sensitive rod outer segments (ROS) are composed of stacks of double membrane 'disks' surrounded by a plasma membrane. Throughout life, the ROS membranes are renewed in a highly ordered manner which has made this cell type the model of choice for many studies on membrane synthesis and turnover. Autoradiographical studies have shown that new membrane precursors are synthesised within the rod inner segment, then assembled into new disks at the base of the ROS, displacing previously formed disks away from the cell body<sup>1,2</sup>. An optimum ROS length is maintained



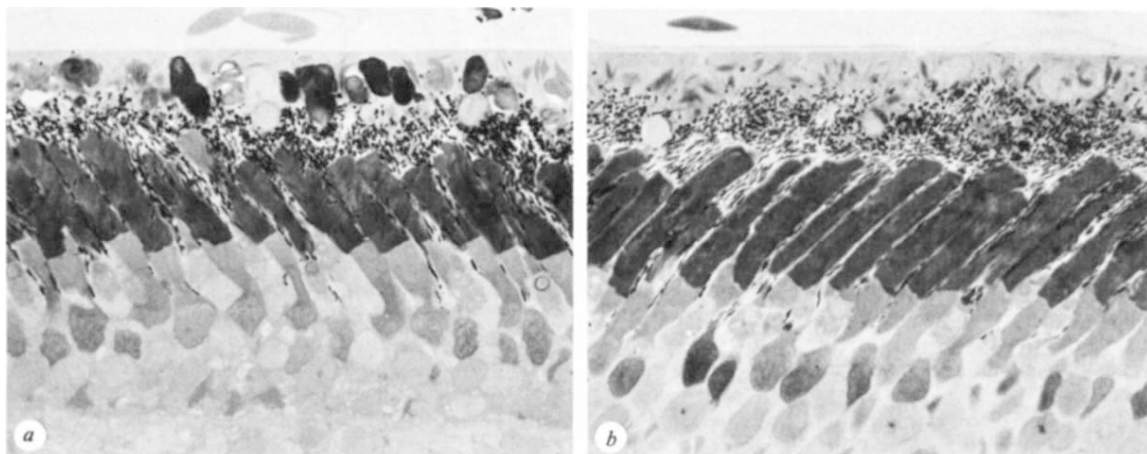


**Fig. 1** Histograms showing the phagosome counts in the pigment epithelium (PE) from frogs maintained in constant light for 5 d. Values are mean counts and bars represent  $\pm 1$  s.e.m. The cartoon illustrates the experimental conditions. When both eyes were covered for 1 h, then uncovered for 2 h, rod shedding was initiated in both eyes as evidenced by the large numbers of phagosomes in the pigment epithelium (left histograms). In a similar manner, when only the left eye was covered, large numbers of phagosomes were observed only in the left eye (centre histograms). When both eyes were left uncovered, only a few phagosomes were present in the pigment epithelium (right histograms).

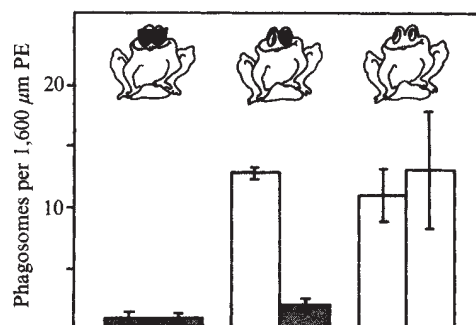
by the periodic shedding of groups of older disks from the ROS tip. These discarded disk packets are then engulfed and degraded by the adjacent pigment epithelium<sup>3,4</sup>. The shed ROS fragments persist for several hours within the cytoplasm of the pigment epithelial cells and are referred to as phagosomes.<sup>3</sup> LaVail<sup>5</sup> has reported that ROS in the rat retina shed their apical tips shortly after the onset of light in animals maintained under cyclic lighting (12 h light (L):12 h dark (D)). We have shown that a similar pattern of rod outer segment shedding occurs in the amphibian retina<sup>6-8</sup>, and similar patterns have also been observed in the goldfish<sup>9</sup>, rabbit<sup>10</sup> and chick<sup>11</sup>. In all these animals, a nearly synchronous burst of ROS shedding occurs within two hours of the onset of light, and minimal amounts of rod shedding occur during the remainder of the day. Also, in amphibians, constant light inhibits daily rod shedding, and the outer segments elongate due to continued

outer segment disk addition<sup>7,12,13</sup>. In the rat<sup>5</sup> and *Xenopus*<sup>7</sup>, shedding occurs at approximately the same time each day in animals maintained for several days in the dark, although in *Rana* the onset of light is required to initiate shedding, and dark-maintained animals do not shed for several days<sup>6</sup>. Taken together, these results have suggested that ROS shedding follows a circadian rhythm related to the onset of the light cycle. The photoreceptive nature of the amphibian pineal gland, and the well-documented circadian rhythm in serotonin-melatonin metabolism in the rat pineal, made this organ a particularly good candidate for controlling photoreceptor shedding, although several other extraocular systems have been suggested<sup>14</sup>. However, several laboratories have demonstrated that pinealectomy, hypophysectomy, superior cervical ganglionectomy, and cutting of the optic nerves have no effect on the diurnal pattern of ROS shedding in either the rat or the frog retina<sup>12,15</sup>. Furthermore, we have been unable to affect rod shedding in the frog retina by direct injection of melatonin, serotonin or the  $\beta$ -adrenergic effectors propranolol and isoprenaline. Thus, even though rod outer segment shedding consistently occurs in both eyes and seems to follow a circadian rhythm in darkness, suggesting some form of central regulation, the intraocular control of this response remained a possibility. We report here experiments which strongly suggest that rod shedding in frogs can be initiated within the eye and is not secondarily controlled by a gland or organ elsewhere in the body.

ROS shedding normally occurs in the frog retina between one and two hours after the onset of light, and in frogs maintained under cyclic lighting (14L:10D), about 20% of the outer segments will shed each day. When frogs are maintained in constant light, shedding is prevented, and after five days of constant light, one hour of darkness is sufficient to cause every rod to shed when animals are returned to light. We used this protocol to test for the intraocular control of rod shedding. A group of 11 frogs was maintained in constant light for five days to potentiate their shedding response. The animals were immobilised for about 60 min with the amphibian anaesthetic MS-222 under room illumination. Black, opaque darkroom cloth was used to cover the frogs so that either both eyes (three frogs), the left eye only (five frogs), or neither eye (three frogs) was occluded for a period of one hour, after which time the cloth was removed and the frogs left under normal room illumination for an additional two hours. They were then killed



**Fig. 2** Photomicrographs of the retina-pigment epithelium interface from left (a) and right (b) eyes of the frog *Rana pipiens*. Eyes were fixed in 3% glutaraldehyde in 0.087 M phosphate buffer at pH 7.2 for 24 h, postfixed in 1% osmium tetroxide in the same buffer for 1 h, then infiltrated with epon. Following polymerisation of the plastic, 1- $\mu$ m sections were taken from the posterior pole of both eyes, and the sections were stained with toluidine blue. Note that the pigment epithelium in the left eye contains many large, densely staining phagosomes and that the rods are shorter when compared to the rod lengths in the right eye. The pigment epithelium in the right eye is free of phagosomes. Magnification  $\times 1,200$ .



**Fig. 3** Histograms showing the phagosome counts in the pigment epithelium (PE) from frogs maintained in cyclic light. Values are mean counts and bars represent  $\pm 1$  s.e.m. The cartoon illustrates the experimental conditions. During the dark phase of their cycle either both eyes were covered (left histograms), the left eye only was covered (centre histograms) or neither eye was covered (right histograms). Animals in this condition were then exposed to light for 30 min followed by 1.5 h of darkness. Phagosomes are more numerous in the light-stimulated eyes than in those covered during the period of light exposure.

and the eyes fixed and sectioned for light microscopy. The frogs recovered fully from the anaesthetic during this two-hour period, and our previous experiments had shown that this anaesthetic has no effect on rod shedding.

Figure 1 shows the results of this experiment. In all frogs where both eyes were covered, rod shedding occurred in both eyes as shown by the large number of phagosomes in the pigment epithelium. In all frogs where neither eye was covered, only a few phagosomes were found, and in all frogs where only the left eye was covered, the pigment epithelium in the left eye was filled with recently shed phagosomes, whereas in the right eye only a small number of phagosomes were seen. Figure 2 shows samples from the left and right eyes, respectively, of one of the monocularly covered frogs, demonstrating the dramatic unilateral nature of this response.

To further substantiate the intraocular control (monocular initiation) of shedding, light stimulation was used in a similar protocol. Our previous experiments have shown that 30 min of room illumination (550 lx) is sufficient to initiate rod shedding. Accordingly, 15 cyclic light-conditioned animals were maintained overnight in darkness and anaesthetised with MS-222 under dim red illumination. The animals were divided into three groups of five animals and the darkroom cloth was used to cover frogs so that either both eyes, the left eye only, or neither eye was occluded. The frogs were then given 30 min of room illumination, after which the lights were turned off. The frogs were killed in the dark 1.5 h later. Figure 3 shows the results of this experiment. In all frogs where both eyes were covered during the period of illumination, few phagosomes were seen in the pigment epithelium, indicating that shedding had not taken place. In all frogs where both eyes were exposed to light, the presence of a larger number of phagosomes indicates that normal shedding had occurred in each eye. In all frogs where only the right eye was exposed to light and the left eye was covered, the number of phagosomes was consistently higher in the exposed eye.

These results provide direct evidence that rod outer segment shedding is under intraocular initiation. In constant light-maintained frogs, when a single eye is darkened for a brief period, then exposed again to light, only the darkened eye will shed. When frogs at the end of the dark phase of their normal diurnal cycle are manipulated so that only one eye is exposed to light, shedding occurs only in the light-stimulated eye. The establishment of intraocular initiation of rod shedding indicates that the control of this phenomenon lies within the eye.

We thank T. Hoffman, L. Marroquin, D. Medford, M. Moss and M. Rayborn. This investigation was supported by the Retina Research Foundation, the National Retinitis Pigmentosa Foundation and USPHS grants EY 01406, EY 02362 and EY 02363. The authors are recipients of Research Career Development Awards from the National Eye Institute.

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Received 27 April; accepted 26 June 1978.

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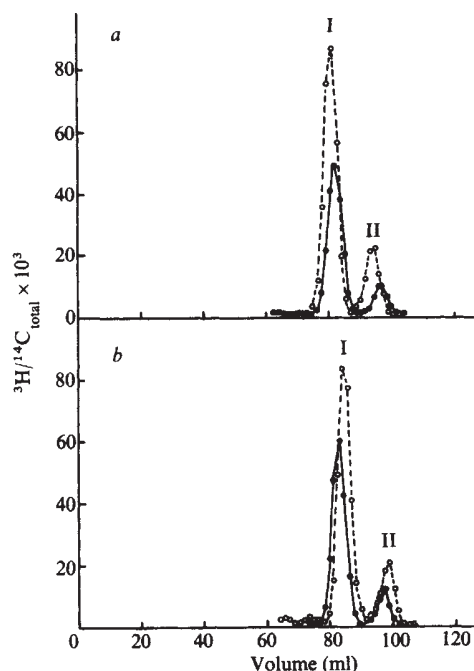
## Excisability and persistence of benzo(a)pyrene DNA adducts in epithelioid human lung cells

RESIDUAL DNA lesions in the template strand during replication may represent the 'stumbling blocks' responsible for mutagenesis and initiation of malignant transformation. The fraction of specific lesions which remains unexcised at the time of DNA replication is determined by their 'excisability'<sup>1</sup> in a particular tissue. Lesions which become refractory to excision and remain in the DNA over many generations, referred to as 'persistent lesions', may play a crucial role in the transformation process<sup>2–4</sup>. We have studied the 'excisability' and 'persistence' of the covalent DNA adducts produced in the epithelioid human alveolar tumour cell A549 (ref. 5) by benzo(a)pyrene (B(a)P)<sup>6</sup>, and found that the excisability of the covalent B(a)P–DNA adducts in A549 cells was poor and that a sizeable fraction of the lesions persisted over several generations. The poor reparability by excision of these and related lesions in human lung cells may represent a factor in lung carcinogenesis by polycyclic aromatic hydrocarbons.

A549 cells were cultured in monolayers, labelled in their DNA with <sup>14</sup>C-thymidine and treated with <sup>3</sup>H-B(a)P for 48 h at a final concentration of 1.3  $\mu$ M as described previously<sup>6</sup>. Treatment with B(a)P at this concentration caused a transitory slight decrease in the rate of growth of A549 cells but had no effect on their colony-forming ability. After different lengths of post-treatment incubation up to 72 h, at which time the cultures were essentially confluent, the level of covalent DNA–B(a)P adducts was determined in digests of total DNA and of the micrococcal nuclease resistant nucleosomal core DNA as described in Fig. 1. As shown in Fig. 1, two major <sup>3</sup>H-containing B(a)P–DNA adduct peaks are formed in A549 cells. Peak I contains exclusively (7R)N<sup>2</sup>-(10-{7 $\beta$ ,8 $\alpha$ ,9 $\alpha$ -trihydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene}yl)-deoxyguanosine (B(a)P-diol-epoxide I-dG); approximately 80% of the second peak consists of (7R or 7S) N<sup>2</sup>-(10-{7 $\beta$ ,8 $\alpha$ ,9 $\beta$ -trihydroxy-7,8,9,



10-tetrahydrobenzo(a)pyrene)yl)deoxyguanosine (B(a)P-diol-epoxide II-dG)<sup>7</sup>. The remaining 20% of peak II has not been structurally identified but most likely consists of other stereoisomeric deoxyguanosine-B(a)P adducts. These structural assignments were made by comparing the mobility of the DNA adducts formed in A549 on Sephadex LH20 columns with adducts produced by the treatment of bacteriophage T7 DNA<sup>8</sup> and A549 cells<sup>9</sup> with chemically synthesised radioactive racemic



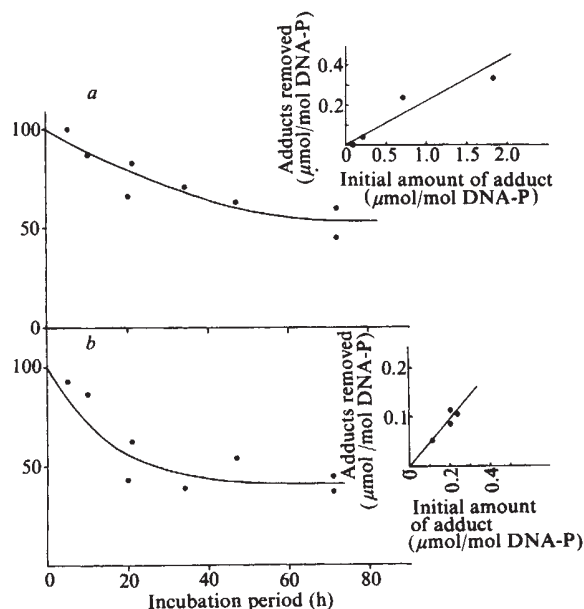
**Fig. 1** Sephadex LH20 chromatograms of DNA digests from A549 cells that had been exposed to <sup>14</sup>C-thymidine and <sup>3</sup>H-B(a)P and then incubated for 0 and 72 h. *a*, Chromatograms of digests of total DNA; *b*, digests of micrococcal nuclease-resistant nucleosomal core DNA. Alveolar epithelial tumour cells (A549) were obtained from Dr S. Brown and cultured in T-150 Corning flasks using Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (Gibco). Cells were plated at  $1 \times 10^6$  per T150 and labelled with <sup>14</sup>C-thymidine ( $1.6 \mu\text{Ci ml}^{-1}$ ) in the presence of  $10^{-6}$  M thymidine. <sup>14</sup>C-thymidine was added every 24 h for a total of three additions. During the last 48 h <sup>3</sup>H-B(a)P ( $24 \text{ Ci mmol}^{-1}$ , Amersham Searle) was added at a final concentration of  $1.3 \mu\text{M}$ . After two washings the treated cultures were incubated in fresh medium for different lengths of time. Cells were then collected and frozen as pellets. Nuclei were prepared from the thawed cells by homogenisation in 5 ml 0.25 M sucrose, 0.01 M Tris (pH 8.0), 0.01 M Mg and 0.5% Triton X-100 with a Dounce homogeniser using the 'B' pestle. Nuclei were washed and resuspended at  $2 \times 10^7 \text{ ml}^{-1}$  in 0.25 M sucrose, 0.001 M Tris (pH 8.0) and 0.0001 M  $\text{Ca}^{2+}$ . Half of the nuclei of each time point was digested with micrococcal nuclease at  $5 \mu\text{g ml}^{-1}$  for 50 min at  $37^\circ\text{C}$  according to Söllner-Webb and Felsenfeld<sup>13</sup>. To stop the reaction excess EDTA was added and the DNA precipitated with 95% ethanol. The ethanol-precipitable core DNA was extracted, enzymatically hydrolysed and the nucleoside mixture chromatographed on Sephadex LH-20 as described previously<sup>11</sup>. The other half of the nuclei preparation received the same treatment with the exception that the pretreatment with micrococcal nuclease was omitted. All <sup>14</sup>C-radioactivity was eluted by the 30% aqueous methanol wash and represented <sup>14</sup>C-thymidine (not shown). Only small amounts of tritium were detected in these initial fractions. The ratios of <sup>3</sup>H-radioactivity contained in each fraction to total <sup>14</sup>C-radioactivity eluted from the column in the 30% aqueous methanol wash are plotted. Peak I contains exclusively B(a)P-diol-epoxide I-dG and peak II contains approximately 80% B(a)P-diol-epoxide II-dG. ○, 0 h post-treatment incubation; ●, 72 h post-treatment incubation.

7β,8α-dihydroxy-9α,10α-epoxy-and-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene and by comparison with authentic samples of B(a)P-diol-epoxide I-dG using high pressure liquid chromatography.

Sephadex LH20 chromatograms of digests of total DNA and micrococcal nuclease-resistant nucleosomal core DNA of A549 cells which had been treated with <sup>3</sup>H-B(a)P and then incubated for 0 and 72 h are shown in Fig. 1*a* and *b*, respectively. The ratios of <sup>3</sup>H-radioactivity contained in each fraction to total <sup>14</sup>C-radioactivity eluted from the column are plotted. The <sup>14</sup>C-radioactivity belongs to <sup>14</sup>C-thymidine and elutes in the 30% aqueous methanol wash of the column (not shown). Ratios of <sup>3</sup>H/<sup>14</sup>C are used to relate the lesion concentration to the amount of DNA present during B(a)P treatment. The <sup>3</sup>H/<sup>14</sup>C ratios of peaks I and II, 0.302 and 0.090 for total DNA and 0.296 and 0.079 for nucleosomal core DNA, are comparable at 0 h post-treatment incubation.

This result indicates that there are equal initial concentrations of B(a)P-diol-epoxide I-dG and B(a)P-diol-epoxide II-dG, respectively, in total and nucleosomal core DNA and consequently also in nucleosomal core and nucleosomal linker DNA. In contrast to our results with intact A549 cells, binding of B(a)P occurred preferentially to nucleosomal linker DNA in isolated calf thymus nuclei which were incubated with B(a)P in the presence of NADPH and rat liver microsomes<sup>10</sup>. Preferential introduction of lesions into nucleosomal linker DNA was also observed after treatment of A549 cells with the highly reactive metabolite 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene. The initial adduct content was two-fold higher in linker than in core DNA (G. F. and P. C., unpublished). The differences in these results are not surprising. In the case of the exposure to a procarcinogen, as in our present

**Fig. 2** Kinetics of removal of DNA-B(a)P adducts from total DNA of A549 cells. *a*, Removal of B(a)P-diol-epoxide I-dG; *b*, removal of B(a)P-diol-epoxide II-dG. Experimental conditions as described in Fig. 1. Inset to *a*, amount of B(a)P-diol-epoxide I-dG (peak I) removed from total DNA within the first 20 h of the post-treatment incubation as a function of the initial lesion concentration. The initial lesion concentration was calculated from the specific activity of <sup>3</sup>H-B(a)P, the DNA content of A549 cells ( $1.7 \times 10^{-11}$  g per cell) and the recovery of DNA by extraction. Inset to *b*, amount of B(a)P-diol-epoxide II-dG (peak II) removed from total DNA within the first 20 h of the post-treatment incubation as a function of the initial lesion concentration.



experiments, the lesion distribution may undergo continuous changes, as it depends on the relative rates of several overlapping metabolic processes whereas the relative chemical reactivity of DNA in different parts of chromatin is the determining factor for the treatment with ultimate carcinogens.

The initial concentration of B(a)P-diol-epoxide I-dG varied considerably, over 0.20–1.84  $\mu\text{mol}$  per mol DNA-P in different experiments, whereas the concentration of B(a)P-diol-epoxide II-dG remained in a narrow range of 0.20–0.31  $\mu\text{mol}$  per mol DNA-P. Variations in the absolute and relative amounts of B(a)P adducts formed have been observed in baby hamster kidney cells, secondary mouse embryo fibroblasts<sup>11</sup> and hamster embryo cells<sup>12</sup>. Changes in the different branches of the metabolic pathways leading to the stereoisomeric B(a)P-diol-epoxide intermediates, their relative biological stability and the rates of excision of the DNA adducts due to slight differences in the growth conditions of the cultures, may explain these observations.

The  $^3\text{H}/^{14}\text{C}$  ratios decreased as a function of post-treatment incubation, indicating selective removal of the adducts from high molecular weight DNA. As shown for the 72 h incubation in Fig. 1, the ratios for the two adduct peaks remained comparable for total and nucleosomal core DNA throughout post-treatment incubation, indicating that adduct removal occurred with similar efficiency from the entire DNA. The time-courses for removal of B(a)P-diol-epoxide I-dG and B(a)P-diol-epoxide II-dG are shown in Fig. 2a and b, respectively. Excision slows down considerably at late times and essentially stops with prolonged incubation for 72 h, which corresponds to approximately three population doubling times of A549 cells. The remaining 'persistent' fraction of lesions after 72 h is approximately 0.55 for B(a)P-diol-epoxide I-dG and 0.40 for B(a)P-diol-epoxide II-dG. From the inserts to Fig. 2, it is evident that the excision capacity of A549 cells was not saturated at the initial lesion concentrations in our experiments since linear relationships were obtained between the amount of lesions initially present in the DNA and the amount removed within the first 20 h of incubation.

Our results show that actively metabolising A549 cells, which were treated with B(a)P over a prolonged period in non-toxic conditions, possessed only limited capacity for removal of covalent DNA-B(a)P adducts from their DNA. Persistent lesions remained in the DNA 72 h post-treatment despite the fact that the excision capacity of the cells earlier during incubation was not saturated. The persistent lesions and the lesions which were removed earlier during incubation were chemically and biochemically indistinguishable: the first peak of the Sephadex LH20 chromatograms contained exclusively B(a)P-diol-epoxide I-dG, whereas the second peak was mostly B(a)P-diol-epoxide II-dG; the lesions remained equally distributed between nucleosomal core and nucleosomal linker DNA during post-treatment incubation and no evidence was obtained for an enrichment of persistent lesions in either DNA fraction. The values for the persistent fraction of lesions varied somewhat for individual experiments. Although no correlation between cell density and lesion persistence was observed, the possibility that the depletion of a nutritional factor could be responsible for our results is being explored.

The most straightforward alternative interpretations of our results are that A549 cells lose their capacity for removal of B(a)P-DNA adducts altogether with continued post-treatment incubation or that the adducts become part of a portion of DNA which cannot be repaired by the excision pathways (but is indistinguishable from total DNA in our conditions of digestion with micrococcal nuclease). An indication for the formation of a persistent fraction of B(a)P adducts was also observed in our earlier studies with secondary mouse embryo fibroblasts but not with baby hamster kidney cells<sup>11</sup>. Persistent B(a)P-DNA lesions in A549 cells may be bypassed by the DNA replication enzymes at late times during post-treatment incubation<sup>9</sup>.

We thank Dr D. Grunberger and his colleagues for the analysis of our samples by high pressure liquid chromatography,

and M. Bravo and A. Lusby for assistance. This work was supported by a USPHS grant GM18617 and contract EY-76-S-05-4155 from the US Department of Energy.

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## Interferon affects both G<sub>1</sub> and S + G<sub>2</sub> in cells stimulated from quiescence to growth

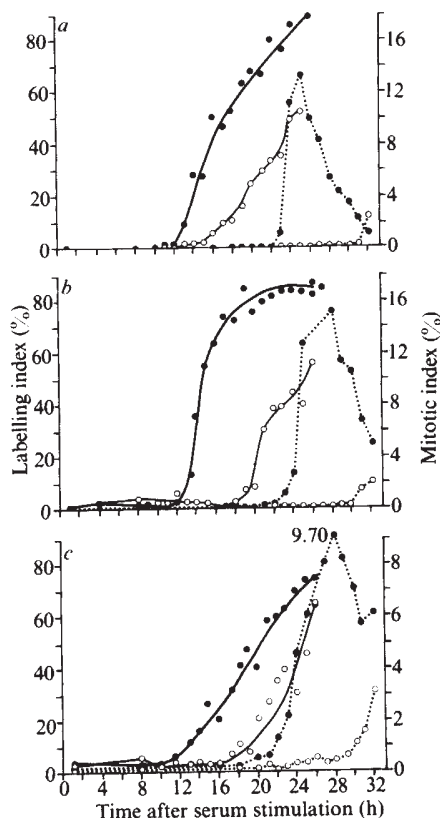
THE interferons were originally described as proteins, produced by cells in response to virus infection, which could induce viral resistance in cells of the same species<sup>1</sup>. It is becoming clear that these proteins have other important biological activities<sup>2</sup> and can inhibit cell division in both normal and malignant cells<sup>3–5</sup>. Although the phenomenon of growth inhibition by interferon is well established, data analysis of the effect in terms of the cell cycle is limited and not completely consistent. Killander *et al.*<sup>6</sup> and Matarese and Rossi<sup>7</sup>, using asynchronously growing cultures of L1210 and Friend leukaemia cells respectively, have found that interferon-treated cells are delayed in both G<sub>1</sub> and G<sub>2</sub>, and we have recently observed a similar effect of interferon on asynchronously growing MCF-7 cells<sup>8</sup>. Sokawa *et al.* have reported, however, that the only parameter affected in interferon-treated quiescent BALB/c 3T3 cells after serum stimulation, is their rate of entry into S (ref. 9). It is possible that the effect of interferon on the cell cycle of asynchronous transformed cells is different, in terms of cell cycle parameters, from its effect on quiescent untransformed cells. In an attempt to clarify this point, we have examined in detail the effect of interferon on the passage of serum-stimulated cells through the cell cycle, using BALB/c 3T3 and Swiss 3T3K cells, and a strain of human embryo lung diploid fibroblasts, HEL 27. In contrast to Sokawa *et al.*, we have found that both G<sub>1</sub> and S + G<sub>2</sub> are extended by interferon treatment of all three cell types studied.

In the experiments reported here, cells were synchronised in G<sub>0</sub>/G<sub>1</sub> by culturing in medium containing low or no serum and then stimulated to grow with 10% serum in the presence or absence of 10<sup>3</sup> U interferon per ml. Tritiated thymidine was added with the serum and plates fixed and processed for autoradiography at hourly intervals to determine the time and rate of entry into S. Unlabelled monolayers were also processed and stained at regular intervals to determine the onset



and rate of entry into mitosis. Figure 1 shows the results obtained using BALB/c 3T3 cells, Swiss 3T3K cells and HEL27 fibroblasts.

Qualitatively similar effects of interferon were observed in all three cell types, although some quantitative differences were



**Fig. 1** Effect of interferon on the entry into S phase and mitosis of serum-stimulated synchronized BALB/c 3T3 cells (a), Swiss 3T3K cells (b) and HEL27 fibroblasts (c). Cells were grown in 30-mm plastic Petri dishes (Nunc, Gibco Biocult) at 37°C in a 10% CO<sub>2</sub> humidified atmosphere and were free of mycoplasma as assessed by staining and growth on agar. BALB/c and Swiss 3T3K cells were synchronized by growing to confluence in medium RPM1 1640 (Gibco Biocult)+10% fetal bovine serum (FBS; Gibco Biocult) and then for 24 h in RPM1 1640 containing 0.5% medium from the confluent cultures (designated med-RPM1 1640+0.5% 'spent' FBS). HEL27 fibroblasts (plated at 10<sup>5</sup> cells per dish) were grown for 4–6 d in med-RPM1+0.5% 'spent' FBS and then in med-RPM1 1640 alone for 24 h. All cultures were stimulated with fresh med-RPM1 1640+10% FBS with or without 10<sup>3</sup> reference units (U ml<sup>-1</sup>) interferon. For measurement of labelling index [methyl-<sup>3</sup>H]thymidine (20 Ci mmol<sup>-1</sup>, 5 µCi ml<sup>-1</sup>, Radiochemical Centre) was added at the time of serum stimulation, and at intervals the dishes were fixed in formal saline and processed for autoradiography, as described previously<sup>11</sup>. Approximately 500 cells were counted per dish to estimate the labelling index. For measurement of mitotic index, cells were swelled by addition of trisodium citrate followed by distilled water and fixed with Carnoy's fixative (absolute alcohol:glacial acetic acid 3:1). Air-dried dishes were stained with aceto-orcin (R. A. Lamb), and ~1,000 nuclei per dish were examined for mitoses. Human interferon was calibrated against MRC research standard B, 69/19 (Natn. Inst. Biol. Stds. Controls, London). Mouse interferon units refer to international reference units. The interferon used in the BALB/c and Swiss 3T3K experiments was mouse L cell interferon (specific activity 5×10<sup>5</sup> U mg<sup>-1</sup> protein; (M. Johnson, Wellcome). Human namalva lymphoblastoid cell interferon (specific activity 3×10<sup>7</sup> U per mg protein) was used in the HEL27 experiment (K. Fantes, Wellcome). This interferon has been shown to have both fibroblast and leucocyte interferon activities<sup>12</sup>. ●—●, labelling index control cells; ○—○, labelling index cells treated with interferon 10<sup>3</sup> U ml<sup>-1</sup>; ●—●—●, mitotic index control cells; ○—○—○, mitotic index cells treated with interferon 10<sup>3</sup> U ml<sup>-1</sup>.

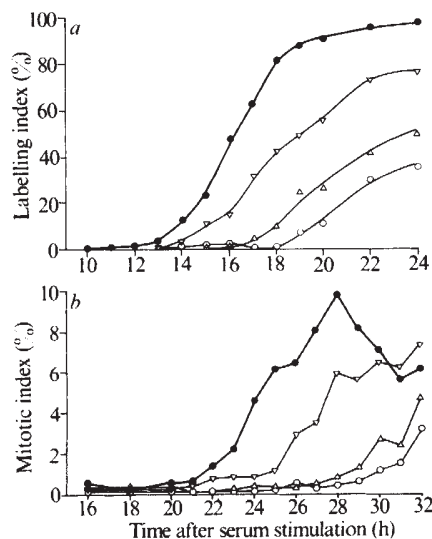
found. From Fig. 1 it can be seen that interferon induced a delay both in entry into S and in entry into mitosis of BALB/c and Swiss 3T3K cells and in HEL27 fibroblasts. If only the G<sub>1</sub> phase of the cell cycle were affected, the delay of entry into S and into mitosis should be the same. In fact, there is a greater delay in entry into mitosis, indicating an effect on S+G<sub>2</sub>, as well as on G<sub>1</sub>. Interferon treatment increases the length of S+G<sub>2</sub> by 7 h in BALB/c 3T3 cells and by 2–3 h in Swiss 3T3 cells and HEL27 fibroblasts. The interferon-associated effects on entry into S and mitosis were dose dependent, and representative data are shown in Fig. 2.

Examination of Fig. 1 shows that while interferon has some effect on the rate of entry into S of BALB/c 3T3 cells, there is also an effect on the lag before S. This delay in the time of entry into S is more marked with Swiss 3T3 cells and HEL27 fibroblasts. The delay in the onset of S and the lengthening of S+G<sub>2</sub> noted by us in interferon-treated BALB/c 3T3 cells appears to conflict with the results of Sokawa *et al.*<sup>9</sup>. This discrepancy may be related to the fact that their BALB/c 3T3 cells seem to be relatively insensitive to interferon compared with the BALB/c 3T3 cells used in our experiments, suggesting there has been selection of different species in culture.

The effect on S+G<sub>2</sub>, which was most marked in BALB/c 3T3 cells, was clearly demonstrated by the experiment shown in Fig. 3, in which interferon was added at the G<sub>1</sub>/S boundary, that is, 11.5 h after serum stimulation. The interferon-induced delay in entry into mitosis was 6 h, compared with 8 h when it was added with the serum. With HEL27 fibroblasts, which showed a marked effect on entry into S and a smaller extension of S+G<sub>2</sub> after interferon treatment, the delay in entry into mitosis was reduced to 1 h when it was added 11.5 h after serum stimulation.

The interferon-induced delay in the entry of all these cells into mitosis was found to be a true lag and not just a change in

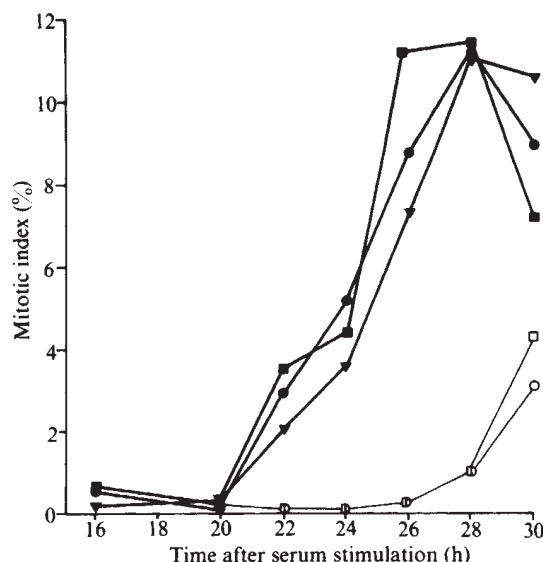
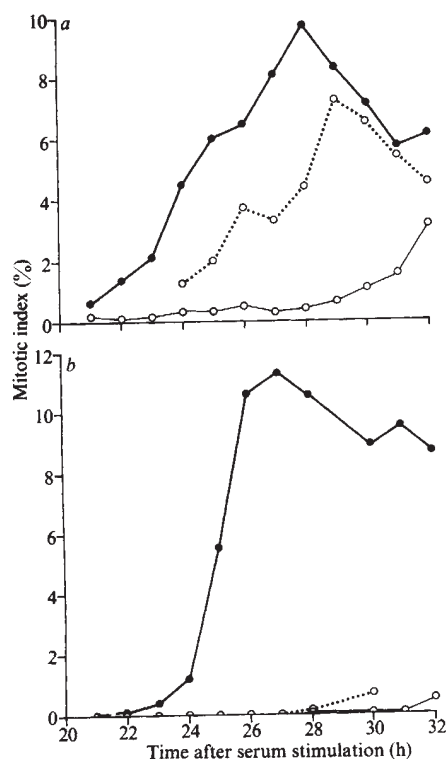
**Fig. 2** Effect of different doses of interferon on the entry of Swiss 3T3K cells (a) into S phase and HEL27 cells (b) into mitosis. Quiescent Swiss 3T3K cells and HEL27 fibroblasts were stimulated with serum and interferon at various concentrations added at the time of serum stimulation. a, Labelling indices of the Swiss 3T3K cells at various times after serum stimulation estimated as described in Fig. 1. The interferon used was mouse L cell interferon (specific activity 5×10<sup>5</sup> U mg<sup>-1</sup> protein). b, Mitotic indices of the HEL27 cells estimated as described in Fig. 1. The interferon used was human lymphoblastoid interferon specific activity 3×10<sup>7</sup> U per mg protein. ●, Control cells; ▽, cells treated with interferon 10<sup>2</sup> U ml<sup>-1</sup>; △, cells treated with interferon 10<sup>3</sup> U ml<sup>-1</sup>; ○, cells treated with interferon 10<sup>4</sup> U ml<sup>-1</sup>.



rate, as the same result was obtained when colchicine was added during late  $G_1$  and S to accumulate mitoses, or when larger numbers of cells per dish (up to  $10^5$ ) were screened when estimating mitotic indices.

Several control experiments showed that the effects observed were attributable to interferon and not to spurious contaminants of the interferon preparations. First, the same effect on time of onset of S and mitosis was observed with preparations of interferons of different purities: for BALB/c 3T3 cells compare the mitotic lag in Fig. 1a (with interferon specific activity  $5 \times 10^5$  U per mg protein) and Fig. 3b (interferon specific activity  $3 \times 10^7$  U per mg protein), and for HEL27 cells compare the mitotic lag in Fig. 1c (interferon specific activity  $3 \times 10^7$  U per mg protein) and Fig. 4 (interferon specific activity  $4.7 \times 10^6$  U per mg protein). Second, the effect was species specific; human interferon had no effect on the time of entry into mitosis of BALB/c 3T3 cells, although the rate of entry was slightly lower (data not shown) and mouse interferon had no effect on human cells (Fig. 4). Moreover, the effect of mouse interferon on Swiss or BALB/c 3T3 cells could be abolished by heating for 3 min at  $100^\circ\text{C}$  (data not shown). As shown in Fig. 4, preincubation of the human interferon with a specific rabbit anti-human interferon serum completely removed the effects observed on mitosis, whereas normal rabbit serum had no effect.

**Fig. 3** Effect of adding interferon at the beginning and end of the  $G_1$  phase on the entry into mitosis of quiescent, serum-stimulated HEL27 fibroblasts (a) and BALB/c 3T3 cells (b). Quiescent serum-deprived BALB/c 3T3 cells and HEL27 fibroblasts were stimulated with serum as described in Fig. 1 and interferon  $10^3$  U  $\text{ml}^{-1}$ , was added at the time of serum stimulation or 11.5 h after, and mitotic indices estimated at the appropriate time intervals as described in Fig. 1. The human interferon used in Fig. 2a had a specific activity of  $3 \times 10^7$  U per mg protein (from K. Fantes, Wellcome) and the mouse L cell interferon used in Fig. 2b had a specific activity  $2-3 \times 10^7$  U per mg protein (from K. Paucker, Medical College of Philadelphia). ●, Control cells; ○—○, cells treated with interferon  $10^3$  U  $\text{ml}^{-1}$  added at 0 h; ○---○, cells treated with interferon  $10^3$  U  $\text{ml}^{-1}$  added 11.5 h.



**Fig. 4** Controls showing the species and interferon specificity of the mitotic lag found with synchronised, interferon-treated HEL27 fibroblasts. Quiescent HEL27 fibroblasts were stimulated with serum as described in Fig. 1, and interferon  $10^3$  U  $\text{ml}^{-1}$ , or control preparations, added at 0 h. Mitotic indices were estimated as described in Fig. 1. Mouse L cell interferon (specific activity  $5 \times 10^5$  U per mg protein) and human lymphoblastoid interferon (specific activity  $4.7 \times 10^6$  U per mg protein) were used in the experiment.  $10^3$  units of the human interferon were preincubated for 1 h at  $37^\circ\text{C}$  with  $10 \mu\text{l}$  specific anti-human interferon serum (K. Fantes) which completely removed all the antiviral activity of the preparation, or  $10 \mu\text{l}$  normal rabbit serum (NRS). ●, Control cells; ▼, cells treated with mouse interferon  $10^3$  U  $\text{ml}^{-1}$ ; ○, cells treated with human interferon  $10^3$  U  $\text{ml}^{-1}$ ; ■, cells treated with human interferon  $10^3$  U  $\text{ml}^{-1}$  preincubated with anti-interferon serum; □, cells treated with human interferon  $10^3$  U  $\text{ml}^{-1}$  preincubated with NRS.

The results reported here show clearly that quiescent cells treated with interferon while in  $G_1/G_0$  are delayed in their subsequent passage through the cell cycle after serum stimulation. In the mouse cell lines and human strain tested, there is an extension of both  $G_1$  and  $S+G_2$ . Thus, it would seem that both in asynchronously dividing transformed cells, and in untransformed cells stimulated by serum from a quiescent to a growing state, interferon can induce an extension of more than one phase of the cell cycle.

We thank Drs H. Rozengurt, R. Brooks and B. Hill for useful discussions, D. Watling for technical assistance, and the Cell Production Unit for plating out some of the cells.

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Received 24 April; accepted 6 July 1978.

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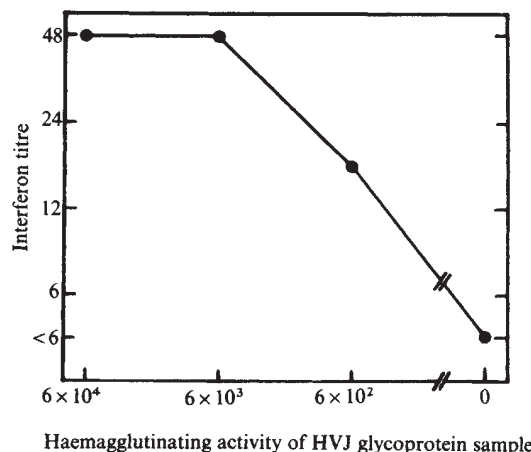


## Active component of HVJ (Sendai virus) for interferon induction in mice

BARON AND BUCKER<sup>1</sup> have shown that circulating interferon is detectable in mice given an intravenous injection of various types of viruses. The interferon production was stimulated by the virus in the inoculum and not by newly replicated viruses, as serum samples assayed for interferon activity were taken at 4.5 h after the intravenous injection, too early for injected viruses to replicate. However, the viral component responsible for interferon induction *in vivo* has not been determined. We describe here our attempts to determine which component(s) of HVJ (Sendai virus) is required for interferon production in mice and report that this activity is found in the surface glycoprotein, particularly in the aggregated form.

Male C57BL/6 mice weighing 25–30 g were used throughout the study. Anti-HVJ haemagglutination inhibition titres in sera of these mice were less than 4. Stock virus of the Nagoya strain of HVJ was prepared by allantoic inoculation of 10-d-old embryonated eggs with 0.1 ml of  $10^{-2}$  or  $10^{-3}$  dilution of infected allantoic fluid. After incubation for 3 d at 35 °C, allantoic fluid was collected and stored at –80 °C. Titration methods of infectivity of the virus for eggs, haemagglutinating activity (HA), haemagglutination inhibitory activity, haemolytic activity and neuraminidase activity have been previously described<sup>2–4</sup>. Interferon was assayed by the cytopathic effects (CPE) inhibition microassay method also previously described<sup>5</sup>, using mouse L cells and vesicular stomatitis virus (VSV) as challenge virus. The highest dilution of a test serum causing at least 50% protection was considered to be the end point. One interferon unit was equivalent to two reference units of mouse interferon.

HVJ preparation partially purified by ultracentrifugation was subjected to ultraviolet irradiation ( $20 \text{ erg mm}^{-2} \text{ s}^{-1}$ ) for 2 h, and then tested for biological activity. As shown in Table 1, infectivity for eggs was completely abolished by this treatment, but other biological activities remained relatively unaffected. One-half of the haemagglutinating activity was retained even after 2 h UV irradiation. Phosphate-buffered saline (0.1 ml, PBS) containing untreated or UV-irradiated HVJ was injected into the tail vein of mice. Eight hours later blood was obtained by cardiac puncture and the serum was then assayed for interferon activity. As shown in Table 1, UV-irradiated HVJ induced as much interferon as did untreated HVJ. This result suggests that the interferon inducer of HVJ is highly resistant to UV irradiation. A similar result has been reported by Youngner *et al.*<sup>6</sup>. The interferon-inducing capacity of Newcastle disease virus in intact mice decreased only slightly over a period of 60 min of UV irradiation. Also, non-infectious virus obtained by heating to 56 °C was capable of inducing a significant level of circulating interferon in intact mice, whereas it was incapable of causing any detectable interferon in chick embryo cell or L cell cultures. As the primary effect of UV



**Fig. 1** Interferon production by isolated viral glycoprotein of HVJ. Mice were given intravenously 0.2 ml of 10-fold dilutions of the glycoprotein sample (starting dose  $6 \times 10^4$  HAU) and 8 h later blood was obtained for assay of interferon activity. Interferon titres were from serum pooled from three mice.

irradiation is on nucleic acid, these results show that the actual inducer of interferon in mice is not viral nucleic acid, but some other viral component(s).

Oxidation by periodic acid, which is known to disintegrate sugars and  $\alpha$ -amino acids, was chosen as a method for abolishing all biological activity except haemagglutination. Mice stimulated with periodate-treated HVJ, which was non-infectious for eggs and without haemolytic and neuraminidase activities, produced a considerable amount of interferon (Table 1). This result indicated that haemolytic and neuraminidase activities were not essential for the virus to induce interferon in mice and that haemagglutinating activity was closely linked to interferon induction.

The above results led us to examine the possibility that isolated viral glycoprotein *per se* may have the ability to induce interferon in mice. HVJ virions were treated with 0.25% (final concentration) NP40 for 15 min at room temperature. The mixture was then centrifuged at 100,000 g for 60 min to remove partially disrupted virions and nucleocapsids, and the supernatant (solubilised envelopes) was dialysed in a Neflex tube against PBS for 7 d to remove NP40. During dialysis the solution containing the solubilised envelopes became turbid as NP40 was removed and membranous particles with spikes were reassembled<sup>7</sup>. These particles had haemolytic, fusion, haemagglutinating and neuraminidase activities. Mice were given intravenously 0.2 ml of 10-fold dilutions of the particle suspension (starting dose  $6.4 \times 10^4$  HA), and 8 h later blood was obtained for assay of interferon activity.

As shown in Fig. 1 and Table 2, circulating virus inhibitor, although of a low titre, was detected in mice given the membranous particles with viral spikes. These membranous particles contained two glycoproteins (HN, F) and little

**Table 1** Interferon production in mice stimulated with HVJ

| Virus                 | EID <sub>50</sub> per 0.1 ml | Haemagglutinating activity | Haemolytic activity ( $\times 40^{-1}$ ) ( $A_{540}$ ) | Neuraminidase activity ( $\times 40^{-1}$ ) ( $A_{540}$ ) | Interferon titre* |
|-----------------------|------------------------------|----------------------------|--------------------------------------------------------|-----------------------------------------------------------|-------------------|
| Untreated HVJ         | $1.6 \times 10^8$            | $4 \times 10^3$            | 0.340                                                  | 0.480                                                     | 3,840             |
| UV-irradiated HVJ     | $<10^1$                      | $2 \times 10^3$            | 0.230                                                  | 0.084                                                     | 3,840             |
| Periodate treated HVJ | $<10^1$                      | $1 \times 10^3$            | 0.000                                                  | 0.008                                                     | 960               |

The virus was UV-irradiated for 2 h, or treated with 0.125 M potassium periodate at 37 °C for 1 h.

\*0.1 ml PBS containing untreated, UV-irradiated or periodate-treated HVJ was injected into the tail vein of mice. Eight hours later blood was obtained by cardiac puncture and the serum was then assayed for interferon activity. Interferon titres were from serum pooled from three mice. Units were the reciprocal of dilution of the serum that gave 50% CPE inhibition.

**Table 2** Interferon production in mice by isolated viral glycoproteins of HVJ

| Inducer                  | Interferon titre* |        |
|--------------------------|-------------------|--------|
|                          | Exp. 1            | Exp. 2 |
| Glycoprotein             | 30                | 48     |
| Glycoprotein + antiserum | 120               | 192    |
| PBS                      | <6                | <6     |

Isolation of envelope protein of HVJ was carried out by the NP40 method. HAU of glycoprotein used in this experiment was  $1 \times 10^4$  HAU. For treatment with antiserum, 0.2 ml glycoprotein sample ( $1 \times 10^4$  HAU) was incubated with 0.2 ml of anti-HVJ envelope protein antiserum (100 HI) at  $37^\circ\text{C}$  for 30 min, and the resulting glycoprotein-antibody complex was then given intravenously to mice. Units were the reciprocal of dilution of the serum that gave 50% CPE inhibition.

\*0.2 ml PBS containing isolated HVJ glycoprotein preincubated with or without antiserum was injected into the tail vein of mice. Eight hours later blood was obtained by cardiac puncture and the serum was then assayed for interferon activity. Interferon titres in the serum were pooled from three mice.

nucleocapsid<sup>7</sup>. The virus inhibitor induced by the membranous particles was nondialysable, stable at pH 2 for 24 h and inhibited CPE formation of VSV in mouse L cells, but not in human embryo lung cells. These findings show that the virus inhibitor is identical to interferon (type I)<sup>8</sup>. Therefore, the above results indicate that the isolated viral glycoprotein(s) of HVJ *per se* have an interferon-inducing ability in mice.

Experiments were then designed to determine whether aggregation of HVJ glycoproteins could enhance their interferon-inducing activity. 0.2 ml glycoprotein sample ( $1 \times 10^4$  HA) was incubated with 0.2 ml of anti-HVJ envelope protein antiserum (100 HI) at  $37^\circ\text{C}$  for 30 min, and the resulting glycoprotein-antibody complex was given intravenously to mice. As shown in Table 2, addition of anti-envelope protein antiserum resulted in the induction of about four times the amount of interferon induced by the viral glycoprotein alone. This finding suggests that aggregation of viral glycoproteins enhances their interferon-inducing activity and that the size of the membranous structure with spikes is related to their interferon inducibility.

De-Maeyer *et al.*<sup>9</sup> reported that interferon induction in mice by HVJ was very sensitive to X-ray irradiation and anti-lymphocyte serum treatment, indicating the involvement of lymphocytes in circulating interferon induction by HVJ. We have previously reported that all the cell populations of the lymphocyte system derived from the mouse spleen are capable of *in vitro* interferon production after stimulation by either untreated or UV-irradiated HVJ<sup>10</sup>. We have also reported<sup>10</sup> that functional viral nucleic acid is required for interferon induction in mouse fibroblast cells, but, in contrast, in mouse spleen cells the viral glycoprotein(s) seems to be the inducer. The results obtained in the present study are consistent with those of De-Maeyer *et al.*<sup>9</sup> and our previous work, and suggest that interferon production in spleen cells and in the intact mouse requires, not the functional nucleic acid needed in fibroblast cultures, but the surface glycoprotein, preferably in aggregated form.

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Received 29 March; accepted 14 June 1978.

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## Clones of cytotoxic lymphocytes can recognise uninfected cells in a primary response against influenza virus

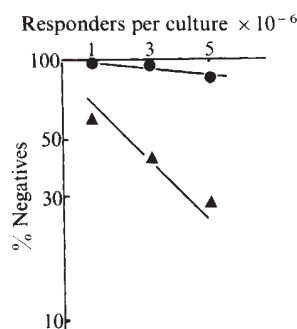
THE specificity of cytotoxic T lymphocytes against influenza virus-infected target cells has been investigated in primary responses *in vivo*<sup>1-5</sup> and in secondary responses both *in vivo*<sup>1</sup> and *in vitro*<sup>2,4,6</sup>. Cold target inhibition techniques have been used to analyse the specificity of responses but this approach has not produced total agreement between all workers<sup>2,3,5,6</sup>. By analogy with antibody production, the most effective way of analysing the specificity of a response is to assess the specificity of a representative sample of monoclonal cytotoxic lymphocytes (CL). To this end we have devised culture conditions whereby individual clones of CL may be generated in a primary antiviral response against influenza A-infected cells. Our work shows that a clonal analysis of virus-induced cytotoxic T cells is feasible. The analysis of individual clones will allow any cross-reactivity towards cells infected with different strains of virus, for example, to be attributed to cross-reactive clones or the existence of several clones each with unique specificity. We report here that in establishing this approach, we have detected several clones which lyse uninfected target cells and we have studied the time of appearance of such clones.

A polyacrylamide culture vessel with a dimpled surface<sup>7</sup> was used to segregate individual clones. Cultures were set up as described previously<sup>8,9</sup>. Spleen cells were obtained from 12-week-old CBA/J mice and were treated with 0.184 M  $\text{NH}_4\text{Cl}$  to remove mouse red blood cells (RBC) before culture. Stimulator cells were taken from spleens from cyclophosphamide-treated mice (200 mg per kg) 5 h after drug injection. Type A/Jap virus stocks ( $\text{A}_2/\text{Jap}/305/57$  ( $\text{H}_2\text{N}_2$ )) were stored frozen as allantoic fluid, and stimulator cells were infected at a ratio of 10 chick RBC-haemagglutination units (HU) to  $10^7$  spleen cells in 0.5 ml for 90 min at  $37^\circ\text{C}$ . L929 ( $\text{H}-2^b$ ) cells were used as targets and were infected in the ratio of 50 HU virus to  $5 \times 10^6$  cells in 0.55 ml.

$8 \times 10^6$  stimulator cells were cultured with various numbers of responder cells and the number of dimples containing cytotoxicity was measured after 5 d, the peak of the response. Both virus-infected and uninfected L929 cells were used as targets and the frequency of precursors or 'clone-forming' cells was derived from the data plotted in Fig. 1.

Using the method of Quintans and Lefkovits<sup>10</sup>, the frequency of antiviral precursors, in relation to the total nucleated spleen cell population, was  $1.9 \times 10^{-5}$ . In addition, a small number of clones which lysed uninfected targets occurred with a frequency of  $2 \times 10^{-6}$  in the experiment shown in Fig. 1. This frequency may be compared with the results obtained using the same technique for an allogeneic combination in which the precursor frequency was shown to be  $5.5 \times 10^{-4}$  (ref. 8). This can be calculated to be 1-2% of the  $\text{Ly}23^+$  CL progenitor pool. Similarly, if it is assumed that the antiviral cytotoxic cells are derived from the  $\text{Ly}123^+$  pool of cells<sup>11</sup>, the measured frequency of antiviral precursors of  $1.9 \times 10^{-5}$  can be calculated to be approximately  $3 \times 10^{-4}$  in relation to the precursor pool. In other work, minimal estimates of anti-trinitrophenyl precursors have been found to be of the order of  $10^{-3}$ - $10^{-4}$  (ref. 12).





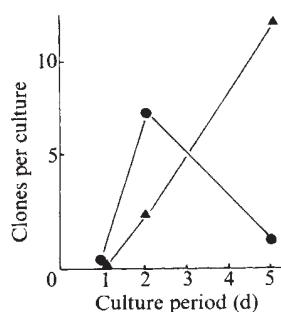
**Fig. 1** Frequency of CL against influenza A/Jap-infected or uninfected syngeneic targets. CBA spleen cells were prepared as described in the text.  $1, 3$  or  $5 \times 10^6$  spleen cells were cultured with  $8 \times 10^6$  syngeneic virus-infected stimulator cells in 'dimpled' polyacrylamide vessels<sup>8</sup>. Quadruplicate cultures were set up for each concentration of responders in RPMI 1640 with 10% heat-inactivated fetal calf serum and  $5 \times 10^{-5}$  M 2-mercaptoethanol. Cytotoxic activity of the cells for each dimple (64 per culture) was examined after 5 d. L929 cells (H-2<sup>k</sup>) were infected with virus for 60 min and  $5 \times 10^6$  cells were labelled with  $100 \mu\text{Ci } ^{51}\text{Cr}$ -chromate in 0.25 ml for 45 min, with two washes between treatments.  $10^4$   $^{51}\text{Cr}$ -labelled cells were mixed with the cells from each dimple in 0.5 ml medium, centrifuged at 100g for 3 min and incubated for 8 h in 5%  $\text{CO}_2$  in air. 0.2 ml of supernatant was removed and  $^{51}\text{Cr}$  release measured. Maximum release was measured after three cycles of freezing and thawing. Spontaneous release was 15–20% for uninfected targets and 20–25% for virus-infected targets. Specific lysis was calculated as:

$$\frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

As described elsewhere<sup>8,9</sup>, cells yielding >10% specific lysis were scored as positive clones.  $\Delta$ , Virus-infected targets;  $\bullet$ , uninfected targets.

The development of clones which lyse uninfected targets was analysed in more detail by splitting the cells from each dimple into two aliquots and assaying each half against either infected or uninfected L cells. As described previously<sup>8</sup>, aliquots were scored as clones when they yielded >10% specific lysis against the appropriate target. Clones were scored as virus-specific if they lysed infected targets and as L929-specific if they lysed both infected and uninfected targets. The frequency of clones was such that the coincidence of more than one clone in a single dimple could be neglected. Figure 2 shows the results of this analysis and both the virus-specific and L929-specific clones

**Fig. 2** The number of CL clones which lyse virus-infected targets or uninfected targets after various periods of culture.  $2 \times 10^6$  CBA spleen cells were cultured with  $6 \times 10^6$  syngeneic virus-infected cells. After 1, 2, or 5 d, the cells from each dimple were suspended in 0.6 ml and split into two equal aliquots.  $5 \times 10^3$  virus-infected or uninfected target cells were added to each half and the chromium release measured as described in Fig. 1.  $\Delta$ , Virus-specific clones;  $\bullet$ , L929-specific clones.



are plotted at each time. The interesting feature of these kinetics is that after 2 d of culture, as many as 75% of the detectable clones lysed uninfected targets.

The nature of the cells in the antiviral response was examined further.  $3 \times 10^6$  CBA spleen cells with  $8 \times 10^6$  virus-infected cells (as above) were set up in 'bulk' cultures<sup>13</sup>. After 2 d in culture the total number of cells from quadruplicate cultures was pooled and divided into four aliquots. The cells were treated with rabbit anti-mouse brain serum (antiThy-1) and complement, with the controls as outlined in Table 1. The treated and control cells were subsequently assayed for cytotoxicity against virus-infected and uninfected L929 cells. Table 1 indicates that the cytotoxicity against both targets was almost completely eliminated by the antiserum, which is specific for T cells.

The detection of CL which lyse uninfected target cells is very similar to the *in vivo* findings of Pfizenmaier *et al.*<sup>14</sup> which described the temporary presence of self-reactive cytotoxic cells during murine lymphocytic choriomeningitis (LCM). Presumably, from a consideration of the pathogenesis of LCM, they suggested that their findings might represent an autoimmune phenomenon. Our results suggest that this phenomenon may be a natural consequence of T cell recognition particularly during a primary response. Irrespective of whether the one-

**Table 1** Sensitivity of cytotoxic cells to antiThy-1 serum

| Treatment              | % Chromium release on day 2* |               |
|------------------------|------------------------------|---------------|
|                        | Uninfected L929              | Infected L929 |
| Antiserum + complement | 2.1                          | 0.9           |
| Antiserum              | 22.5                         | 22.9          |
| Complement             | 39.6                         | 26.2          |
| Untreated              | 29.6                         | 30.8          |

Spleen cells were taken from 2-d cultures and treated with 1/100 dilution of rabbit anti-mouse brain serum for 30 min at 37 °C and with guinea pig serum, absorbed with agarose, for 30 min at 37 °C. One-third of a culture was assayed against  $5 \times 10^3$   $^{51}\text{Cr}$ -labelled target cells.

\* Mean of duplicate assays.

receptor or two-receptor model of recognition is accepted, the system of antiviral recognition can be envisaged as clonally distributed units with a range of affinities. The clones which lyse uninfected cells may represent the subset of cells with low avidity for virus-modified targets but with sufficient avidity to recognise, for example, the histocompatibility antigens of the targets. It could be argued that such low avidity clones might be expected to be detectable early in the primary response. Alternatively, some strains of influenza A have been described as mitogenic<sup>15</sup>, so that if such strains are capable of generating a polyclonal response this would obscure the specificity of 'antigen-stimulated' clones. The demonstration that approximately 10% of 'antigen-stimulated' clones are detected by virtue of their ability to lyse uninfected targets needs to be considered in any future analyses of antiviral specificity.

From a theoretical point of view, it may be possible to exclude some of the current models of T cell recognition by the analysis of clones to examine the immune repertoire in terms of the precursor frequency against a variety of antigens. The clonal analysis of a primary anti-influenza virus response allows the examination of the specificity of individual clones before the primary sample of the repertoire is obscured by the selection of high affinity clones which may occur during a secondary response.

These data show how experimental approaches may be devised to measure the specificity of clones which are generated with a very low frequency. The ability of individual clones to discriminate between cells infected with different strains of influenza A is under investigation and the possibility of extending the doctrine of 'original antigenic sin' to T cell

recognition is clearly important in the study of the efficiency of immunisation and the pathogenesis of viral infections.

This work was supported by the MRC of New Zealand.

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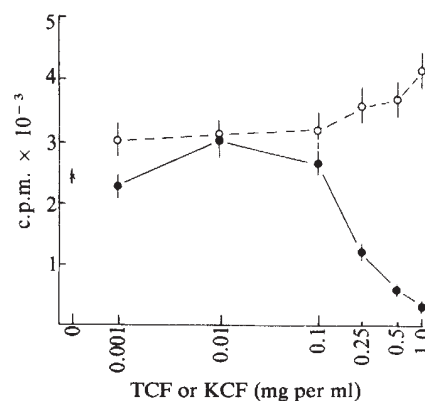
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## Is thymus-derived lymphocyte inhibitor a polyamine?

CELL PROLIFERATION is assumed to be under the control of homeostatic mechanism(s)<sup>1</sup>. Bullough and Laurence<sup>2</sup> introduced the concept of cell-specific endogenous inhibitors of cell proliferation, the so-called chalones. Evidence for the existence in lymphoid tissues of factors meeting the criteria for the definition of a lymphocyte chalone has been reported by several groups<sup>3–6</sup>. The nature of these substances however is ill-defined, since purification from crude extracts of lymphoid tissue (thymus and spleen) has been only partly successful. A lymphocyte inhibitor isolated from bovine thymus tissue has been suggested to be a spermine protein complex<sup>7</sup>: it was quoted that the inhibitory activity on lymphocyte proliferation *in vitro* could only be observed if fetal calf serum (FCS) was present in the culture medium<sup>7</sup>. In contrast to these findings we now report the existence of a nondialysable, active substance extracted from bovine thymus tissue, that inhibits strongly *in vitro* proliferation of human and murine lymphoid cells in the absence of FCS.

Extracts from fresh bovine thymus tissue were made using Kiger's procedure<sup>3</sup> with slight modification. The tissue was homogenised in distilled water (w/v = 1:2) and incubated overnight. The homogenate was centrifuged (1 h at 20,000 g) and the resulting supernatant extracted with Freon 113 (v/v = 1:1). The aqueous phase was collected and cold ethanol (–20 °C) was added to a final percentage of 42% ethanol by volume. The supernatant fraction, after centrifugation, was extensively dialysed against 20 volumes of distilled water and lyophilised (thymus crude fraction, TCF). Following the same procedure, control preparations were obtained from bovine kidney (kidney crude fraction KCF) and liver tissue (liver crude fraction, LCF). Aliquots were dissolved in the appropriate culture medium, sterilised by Millipore filtration (0.45 µm) and stored at –20 °C.

Fractions isolated by this procedure inhibited the spontaneous DNA synthesis of mouse thymocytes in a dose-

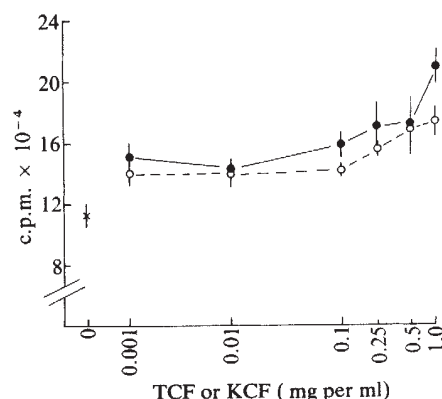


**Fig. 1** Effect of TCF and KCF on spontaneous DNA synthesis of mouse thymocytes.  $10^5$  Swiss thymocytes were cultured in round-bottom microtitre plates (Greiner) in 0.10 ml minimum essential medium-Tris with or without 50 µl sterile fractions for 5½ h at 37 °C and pulsed 3½ h before cell collection with 1 µCi [methyl-<sup>3</sup>H]TdR (5 Ci mmol<sup>-1</sup> Radiochemical Centre Amersham). Cells were collected and precipitable material collected using an automated multiple sample collector (Skatron). Samples were counted in a nuclear Chicago NC 725 liquid scintillation counter. The mean and s.d. of quadruplicate cultures are shown. ●, TCF; ○, KCF.

dependent manner (Fig. 1). No inhibition was observed when TCF was added at various concentrations to mouse mastocytoma cells (Fig. 2). Liver or kidney fractions did not exhibit significant suppressive activity. It should be emphasised that the short-term cultures used for the assay were not supplemented with serum. Addition of FCS to the culture medium did not influence the suppressive activity of TCF. In contrast to TCF, spermine and spermidine in concentrations up to 1,000 µg ml<sup>-1</sup> did not inhibit in a dose dependent fashion the spontaneous DNA synthesis of thymocytes and mastocytoma cells in the absence of FCS in the culture medium, whereas also in the presence of FCS the spontaneous incorporation of thymidine did not decrease below control levels (Fig. 3).

DNA synthesis of mouse spleen cells induced by mitogens (concanavalin A, phytohaemagglutinin and bacterial lipopolysaccharide) could also be blocked with TCF, whereas spermine and spermidine showed only an inhibitory effect at rather high concentrations (Fig. 4). These cultures were normally provided with pooled human AB serum in the culture. On substitution

**Fig. 2** Effect of TCF and KCF on spontaneous DNA synthesis of mouse mastocytoma cells. Mastocytoma cells (P 815-X2 syngeneic to DBA/2) were cultured in Falcon culture flasks in a medium consisting of RPMI 1640 with 25 mM HEPES supplemented with 10% FCS, antibiotics and L-glutamine. After extensive washing with medium,  $10^5$  mastocytoma cells were cultured in a spontaneous proliferation assay as described in Fig. 1. The mean and s.d. of quadruplicate cultures are shown. ●, TCF; ○, KCF.

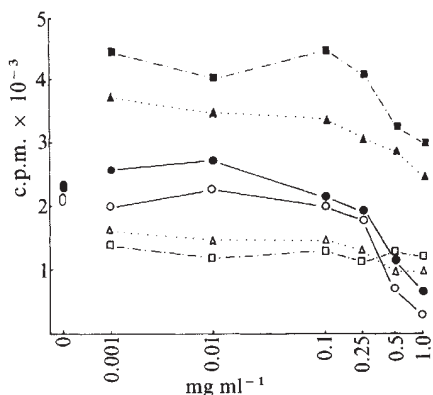




with FCS, polyamines dramatically inhibited induced lymphocyte proliferation (Fig. 4), while no change in the dose-dependent response for TCF was found.

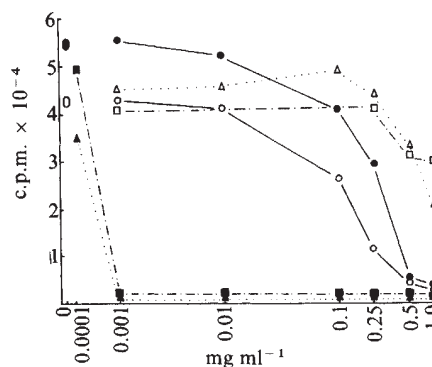
Our results partly confirm previous reports<sup>8</sup> on the essential role of FCS for the full expression of the inhibitory activity of spermine and spermidine on mitogen-induced lymphocyte proliferation *in vitro*.

Interestingly, suppression by both polyamines in the presence of AB serum was less than with FCS. It has been suggested<sup>9,10</sup> that polyamines like spermine and spermidine could be converted by an amine oxidase to aldehydes, substances extremely toxic for mammalian cells in culture. Sheep, calf or fetal sera contain significant amounts of this enzyme in contrast to mouse or human serum<sup>10</sup>. This difference in amine oxidase content between human AB serum and FCS could account for the higher degree of polyamine-induced inhibition observed in the presence of FCS. Note that in our hands the conversion of polyamines into inhibitory moieties was achieved in heat-depleted sera, as it has recently been reported that the conversion of polyamines by human pregnancy serum is based on the presence of (a) heat labile enzyme(s)<sup>11</sup>.



**Fig. 3** Effect of polyamines and TCF on spontaneous DNA synthesis of mouse thymocytes in the presence or absence of FCS.  $10^5$  Swiss thymocytes were cultured in a medium supplemented with or without 5% heat-depleted FCS (Gibco). Spermidine as the tri-hydrochloride and spermine as the tetra-hydrochloride (Sigma) were dissolved in serum-free medium, Millipore-sterilised and added at various concentrations to the cultures. The means of quadruplicate cultures are shown; s.d. did not exceed 10–12% and for reasons of clarity is not shown. Results are presented of cells in the absence (open symbols) and presence (solid symbols) of 5% FCS. ○, ●, TCF; △, ▲, spermine; □, ■, spermidine.

In contrast to the above observation, no serum-dependent change in the inhibitory potency of TCF on mitogen-induced lymphocyte proliferation was found (Fig. 4). Also, no difference in the inhibitory effect of TCF was noted in short-term murine thymocyte cultures, whether 5% FCS was present or not. It is unlikely therefore that the inhibitory activity of TCF against lymphocyte proliferation is based on the presence of polyamines in these partly purified thymus extracts. One could still argue however, that the presence of aldehyde derivatives of spermine and spermidine as such, present as a contaminant in TCF, could account for the inhibition of proliferation. Indeed, it is difficult, if not impossible, to rule out completely this possibility. However, deamination of spermine and spermidine leads to complex labile aminoaldehydes<sup>12</sup>, that most probably will not survive the preparation procedure of TCF. This is in accord with our finding that TCF does not possess cytotoxic properties (data not shown). Furthermore, we could show in this and subsequent studies, which included the use of HeLa cells and human fibroblasts<sup>13,14</sup>, that the inhibitory activity of TCF is cell-specific. This implies that whatever substance might be responsible for the proliferation inhibition, it contains a moiety that determines the cell-specificity. If



**Fig. 4** Serum requirement for polyamine inhibition of mitogen responsiveness.  $3 \times 10^5$  Swiss spleen cells were cultured in 0.05 ml RPMI 1640 with 16 mM  $\text{NaHCO}_3$  buffer medium with 10% heat-depleted pooled human AB serum or 10% heat-depleted FCS plus antibiotics and 2 mM L-glutamine in round-bottom microtitre plates. To each well, 50  $\mu\text{l}$  of a solution containing 10  $\mu\text{g}$  lipopolysaccharide (Difco), then 50  $\mu\text{l}$  of sterile fractions or controls to be tested were added, making a total volume of 0.15 ml. Cultures were incubated in a humidified atmosphere of 5%  $\text{CO}_2$  in air for 48 h, and uptake from 1  $\mu\text{Ci}$  [methyl- $^3\text{H}$ ]TdR (5 Ci  $\text{mmol}^{-1}$ ) was measured for the last 7 h of incubation. The mean of quadruplicate cultures are shown; s.d. was less than 10% and so for reasons of clarity is not shown. Results are presented for cells cultured in the presence of 10% human AB serum (open symbols) or 10% FCS (solid symbols). The polyamines spermine (△, ▲) and spermidine (□, ■) were tested in comparison to TCF (○, ●). Similar curves were obtained for phytohaemagglutinin and concanavalin A.

endogenous aminoaldehydes are responsible for the inhibition, these molecules must by some mechanism exclusively be complexed with a lymphocyte specific carrier. It is unlikely that this could happen *in vivo*. Finally, if polyamines or their conversion products are responsible for the inhibitory effect of TCF, it is not clear why TCF could inhibit spontaneous proliferating lymphoid cells, whereas polyamines (even in the presence of FCS) did not (Fig. 3).

From the experiments described above and other data<sup>3–7</sup>, we conclude that TCF expresses a non-species-specific but lymphocyte-specific inhibitory effect on cell DNA synthesis, which is not based on regulatory effects of aminoaldehydes. In agreement with this conclusion we could demonstrate in a separate study that the inhibition is not based on cytotoxicity, that inhibition is reversible and that TCF also suppresses DNA synthesis in lymphoid tissue *in vivo* when administered to mice. It is worthwhile therefore to attempt further purification of the inhibitory activity.

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Received 2 May; accepted 26 June 1978.

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## ***In vivo* immunisation and reaction against TNP-modified splenocytes**

CYTOTOXICITY tests *in vitro* detect some components of the graft rejection reaction. In one such test, antigenically modified (hapten trinitrophenyl TNP) mouse lymphocytes are detected by the action of cytolytic T lymphocytes (CTL) if the sensitisation of the effector cells is carried out *in vitro*<sup>1</sup>. In the CTL test there is evidence<sup>2</sup> that the reaction is not against the hapten, but against antigenic membrane groups coded by *H-2D* and *H-2K* genes, a phenomenon termed '*H-2* restriction'. In contrast, immunisation *in vivo* with TNP-modified lymphocytes results in a low production of CTL as detected *in vitro*, due probably to production of T suppressor cells<sup>3</sup>. The aim of the present work was to compare the *in vivo* efficacy of the response of the TNP splenocytes with that obtained *in vitro*, and to determine whether the *H-2* restriction applies. Our results show that the immunogenetic implications differed according to whether the test was carried out *in vivo* or *in vitro*, and suggest that the effectors for target cell destruction are also different.

The all *in vivo* method used was that developed by Bainbridge and Gowland<sup>4</sup> for allogeneic reactions, subsequently adapted by Burstein for weaker reactions<sup>5</sup>. Briefly, we injected intravenously aliquots of <sup>51</sup>Cr-labelled, TNP-modified syngeneic splenocytes into mice which had not been immunised (controls), or into mice previously immunised by weekly injections of TNP-modified splenocytes. Two days after the radiolabelled injection, all the mice were killed and their spleens removed and counted for radioactivity in a well-type  $\gamma$  ray counter. The mean c.p.m. for each group was calculated and the value obtained in the controls was taken as 100%. The greater the difference in value from 100% of an experimental group, the more intense was the reaction. Provided that the immunisation was carried out in good conditions, this reaction can be considered to be a sort of graft rejection<sup>4</sup>. From the data shown on Table 1, it seems that TNP-modified syngeneic splenocytes are subject to this kind of *in vivo* reaction, if the recipients were immunised previously two or more times. The reaction is reproducible, necessitates only a few experimental mice and gives results of quantitative significance, without too great a spread in numerical values.

Having established the existence of the *in vivo* reaction against this antigenicity, we then tested whether the *H-2*

restriction is also observed in this case. Genetically similar mice were immunised three times at weekly intervals with TNP-modified allogeneic splenocytes. The reaction was tested against syngeneic TNP splenocytes. Of five different experiments, four gave the *in vivo* reaction, regardless of whether the immunisations had been carried out with TNP-modified cells which were the same as, or different from, those which were tested. In experiments 1 and 2, immunisation with allogeneic *H-2* different TNP splenocytes, resulted in a more intense response than immunisation with syngeneic TNP-modified splenocytes. In experiment 5, however, there was a suggestion that *H-2* restriction could possibly have been operating *in vivo*, because anti-TNP-CBA/Br cells could not elicit a reaction against TNP-CBA/Ca, when TNP-CBA/Ca did so. It is known that CBA/Ca and CBA/Br strains differ by their *H-2* chromosomes<sup>6</sup>, the first being *H-2<sup>k</sup>* and the second one *H-2<sup>d</sup>* (I. Brand, personal communication).

The results of these eight different experiments establish that TNP-modified splenocytes can elicit and develop fully an entirely *in vivo* reaction against syngeneic splenocytes modified in the same way.

Not unexpectedly the *in vivo* results differed in several respects from those obtained with the *in vitro* CTL tests using TNP-modified lymphocytes. We therefore wished to test the possible action of antibodies in the *in vivo* events. Sera of hyperimmunised and control mice were injected into reactive mice a few minutes after the <sup>51</sup>Cr-labelled test cells were injected. Table 3 indicates that cytotoxic antibodies might participate in the observed reaction, but the actions of antisera, compared with those of normal sera, are rather weak, suggesting that the strong reactions observed *in vivo* in actively immunised animals (Tables 1 and 2) are mainly due to cellular reactions. This conclusion agrees with that of Bainbridge and Gowland<sup>4</sup>, who found results comparable with the present results, during a study of the action of serum factors in allogeneic transplantation antigens.

Weak 'natural' antigens (such as *H-Y* (ref. 7), *H-3*, *H-13*, and *H-8* (ref. 8)) have been studied in CTL reactions and were found, in this context, to present the '*H-2* restriction' phenomenon<sup>7-8</sup>, as do the antigens from TNP modification of lymphocytes<sup>2</sup>. In an attempt to discover more about the '*H-2* restriction' phenomenon, Wettstein *et al.* studied *in vivo*, by skin grafting, the reaction against the *H-7* antigen<sup>9</sup>. They found that, instead of the *H-2* restriction which could have been expected, anti-*H-7* skin rejections occurred after immunisation with material containing different *H-2* antigens. The aim of the present work was to test an *in vivo* counterpart

**Table 1** Recovery of <sup>51</sup>Cr-labelled TNP-modified syngeneic splenocytes in the spleens of specifically immunised hosts

| Exp. no. | Mouse strain (sex) | No. of weekly immunisations | No. of mice | Mean splenic c.p.m. | Range of splenic c.p.m. | Recovery in the spleen compared with controls (%) |
|----------|--------------------|-----------------------------|-------------|---------------------|-------------------------|---------------------------------------------------|
| 1        | A.CA (male)        | 0 (controls)                | 5           | 7,346               | 6,813-8,130             | —                                                 |
|          |                    | 2                           | 2           | 4,347               | 4,053-4,642             | 59                                                |
|          |                    | 4                           | 4           | 4,120               | 3,492-5,568             | 56                                                |
|          |                    | 6                           | 4           | 3,094               | 2,723-3,745             | 42                                                |
| 2        | A/Sn (male)        | 0 (controls)                | 4           | 9,240               | 7,894-10,689            | —                                                 |
|          |                    | 3                           | 6           | 3,798               | 2,869-4,789             | 41                                                |
| 3        | A/Sn (female)      | 0 (controls)                | 6           | 9,159               | 6,183-14,626            | —                                                 |
|          |                    | 3                           | 6           | 3,725               | 2,939-4,158             | 40                                                |

Spleen cells were freed from red cells by a brief contact with ammonium chloride. For <sup>51</sup>Cr-labelling,  $1 \times 10^7$ – $3 \times 10^7$  nucleated viable splenocytes were incubated with 400–1,000  $\mu$ Ci of <sup>51</sup>Cr-sodium chromate (from C.E.A.), 200–350 mCi mg<sup>-1</sup>. Incubation was in MEM or RPMI 1640 plus 10% foetal calf serum medium at 37°C, 75 min with occasional shakings. The cells were washed three times before TNP modification. Modification with TNP was carried out with either <sup>51</sup>Cr-labelled cells (for test), or untreated spleen cells (for immunisations). The cells were left for 10 min in a water bath at 37°C in the presence of 10 mM 2,4,6-trinitrobenzene sulphonic acid, without foetal calf serum in the medium. After the incubation, the cells were washed three times in complete medium and used either for intraperitoneal immunisation (in this case, at a ratio of one donor of immunising spleen cells: one immunised mouse), or intravenous challenge (0.2–0.3 ml) with <sup>51</sup>Cr-labelled cells. The number of viable cells was determined in a haemocytometer by the Trypan blue exclusion test before injection. Suspensions with viability of less than 60% were not used.



**Table 2** Recovery of  $^{51}\text{Cr}$ -labelled TNP-modified syngeneic splenocytes in mice immunised against either the same or different TNP-modified splenocytes

| Exp. no. | Reactive mice               | Immunised with                  | Tested with                     | c.p.m. $\pm$ s.d.   | Recovery in the spleen compared with controls (%) |
|----------|-----------------------------|---------------------------------|---------------------------------|---------------------|---------------------------------------------------|
| 1        | B10.D2                      | —                               | TNP-B10.D2                      | 62,041 $\pm$ 20,961 | —                                                 |
|          |                             | TNP-B10.D2                      | TNP-B10.D2                      | 35,095 $\pm$ 4,828  | 56                                                |
|          |                             | TNP-B10                         | TNP-B10.D2                      | 25,777 $\pm$ 4,732  | 41                                                |
| 2        | B10.D2                      | —                               | TNP-B10.D2                      | 3,503 $\pm$ 196     | —                                                 |
|          |                             | TNP-B10.D2                      | TNP-B10.D2                      | 2,727 $\pm$ 197     | 77                                                |
|          |                             | TNP-B10.Br                      | TNP-B10.D2                      | 1,986 $\pm$ 63      | 56                                                |
| 3        | [A.SW $\times$ B10.A(4R)]F1 | —                               | TNP-[A.SW $\times$ B10.A(4R)]F1 | 12,570 $\pm$ 750    | —                                                 |
|          |                             | TNP-[A.SW $\times$ B10.A(4R)]F1 | TNP-[A.SW $\times$ B10.A(4R)]F1 | 7,966 $\pm$ 1,119   | 63                                                |
|          |                             | TNP-B10.D2                      | TNP-[A.SW $\times$ B10.A(4R)]F1 | 8,162*              | 64                                                |
| 4        | [A.SW $\times$ B10.A(5R)]F1 | —                               | TNP-B10.A(5R)                   | 67,698 $\pm$ 12,181 | —                                                 |
|          |                             | TNP-B10.A(5R)                   | TNP-B10.A(5R)                   | 24,381*             | 36                                                |
|          |                             | TNP-A.SW                        | TNP-B10.A(5R)                   | 21,170 $\pm$ 4,540  | 31                                                |
| 5        | CBA/Ca                      | —                               | TNP-CBA/Ca                      | 28,226 $\pm$ 2,261  | —                                                 |
|          |                             | TNP-CBA/Ca                      | TNP-CBA/Ca                      | 18,520 $\pm$ 3,454  | 65                                                |
|          |                             | TNP-CBA/Br                      | TNP-CBA/Ca                      | 27,804 $\pm$ 1,323  | 98                                                |

The same experimental procedures as for Table 1 were used, except that three weekly immunisations were used uniformly and that more variability was introduced in the numbers of  $^{51}\text{Cr}$ -labelled cells injected (this has no effect on the results when expressed as percentages). The number of mice used for each point ranged between 1 and 5. Like the A/Sn and A.CA mice used in Table 1, the A.SW came from Dr Eva Klein, Stockholm; the B10, B10.Br, B10.D2 and CBA/Ca were from the MRC, Carshalton, Surrey; the CBA/Br were given by Dr O. Muhlbock, Amsterdam; B10.A(4R) and B10.A(5R) mice were a gift from Dr Colombani, Paris; and hybrids were made in our laboratories, under standard conditions.

\* One mouse only used.

to the *in vitro* results obtained with TNP-modified lymphocytes, skin grafting obviously not being feasible in this case. The advantages of such a study are the fact that the TNP-modified lymphocytes have been studied widely, and that the hapten under consideration is better defined than the 'natural' haptens against which skin graft reactions are directed.

From our results, it seems that (like Wettstein *et al.*<sup>9</sup>) in most of the cases we observed cross immunisations and not *H*-2 restriction: thus, having the same *H*-2 antigen in the immunising and tested materials is not a prerequisite for the reaction to occur, at least in four out of the five situations studied (Table 2). In particular, experiment 1 of Table 2 shows cross reaction between TNP-B10.D2 and TNP-B10 modified cells, a genetic situation where a strict '*H*-2 restriction' phenomenon was observed *in vitro*<sup>1</sup>.

This apparent lack of *H*-2 restriction could be due to the generation *in vivo* of several (instead of one) restricted populations of cytotoxic lymphoid cells<sup>10</sup>, and this possibility is being tested at present. Also, the cross reactions observed could result from the participation of antibodies in the 'rejection' of the  $^{51}\text{Cr}$ -labelled splenocytes. The results in Table 3 suggest such a possibility, but the relative weakness of the action of antibodies, even when the antiserum donor was immunised six times, indicates the conclusion that the observed reactions probably proceed mainly from cellular reactions<sup>4</sup>.

In addition to the occurrence of frequent cross immunisations, our results show another feature, also shared with Wettstein's results: the *H*-2 antigens which accompany the immunising material seem to influence the intensity of the response. For instance, in experiment 1 of Table 2, immunisation with *H*-2 different TNP-B10 lymphoid cells resulted in a significantly stronger reaction against TNP-B10.D2 targets than did the immunisation with syngeneic TNP-B10.D2 cells. This could be interpreted as an *H*-2 influence on the quantitative expression of the TNP immunogenicity, as in the case of *H*-7 (ref. 9) or *H*-Y (ref. 11) antigens. In the present case, however, because the hapten is presented on intact foreign *H*-2-bearing lymphoid cells, this action could also be explained by the existence of graft-versus-host reaction resulting in the well known 'allogeneic effect'<sup>12</sup>. The use of irradiated lymphoid cells as immunogen carriers will help to determine whether the observed influence of *H*-2 is of the same nature as that observed in the case of skin graftings, or due to a graft-versus-host reaction.

In conclusion, we here propose a functional alternative to the *in vitro* CTL studies of anti-TNP-modified lymphocytes. Even with this defined hapten, some points are still obscure regarding the frequency of occurrence of the *H*-2 restriction phenomenon in CTL, which seems not always absolute<sup>13,14</sup> and might be dependent on the genetic system used<sup>13</sup>. In some

**Table 3** Action of normal and immune sera on TNP-modified syngeneic  $^{51}\text{Cr}$ -labelled splenocytes recovery in spleen of recipients

| Reactive mice | Volume of serum injected* (ml) | No. of weekly immunisations given to serum donors† | Tested with | c.p.m. $\pm$ s.d.  | Recovery in the spleen compared with controls (%) |
|---------------|--------------------------------|----------------------------------------------------|-------------|--------------------|---------------------------------------------------|
| B10.BR        | 0.5                            | 0 (control)                                        | TNP-B10.BR  | 17,290 $\pm$ 4,547 | —                                                 |
|               | 0.5                            | 6                                                  | TNP-B10.BR  | 13,817 $\pm$ 993   | 79                                                |
| C3H           | 0.3                            | 0 (control)                                        | TNP-C3H     | 10,792 $\pm$ 1,151 | —                                                 |
|               | 0.3                            | 3                                                  | TNP-C3H     | 9,432 $\pm$ 617    | 87                                                |

\* These injections were performed intraperitoneally, within 15 min after the intravenous injection of  $^{51}\text{Cr}$ -labelled splenocytes.

† Serum donors, syngeneic with the reactive mice, were immunised with syngeneic TNP-modified splenocytes in the same conditions as described in the legend to Table 1.

cases, recognition of the TNP group itself cannot be excluded, even in the CTL assay<sup>14,15</sup>. Thus, *in vivo* investigations, where effectors other than CTL (namely, antibodies) probably participate, also seem useful. With that aim in view, other workers are comparing CTL assays with *in vivo* investigations, using weak antigens which can be studied by skin grafting<sup>9</sup>. As the method introduced by Bainbridge and Gowland<sup>4</sup> seems to mimic the graft rejection assay, however, we find it useful to apply it to the *in vivo* study of the reaction against TNP-bearing target cells.

This work was supported by INSERM contract ATP 43-76-75. We thank Josiane Bara for preparation of the manuscript, and Françoise Harter, Chantal Beugnot and Pascal Mouchette for technical assistance.

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Received 7 March; accepted 10 July 1978.

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## Cytochalasin D induces the capping of both leukaemia viral proteins and actin in infected cells

It has been reported that cytochalasin B (CB) inhibits a cell-cycle-dependent step of Kirsten murine sarcoma-leukaemia virus maturation<sup>1</sup>. As CB inhibits hexose transport and acts on contractile elements<sup>2</sup>, the inhibition of virus maturation could be due to a hexose deficiency, the disruption of microfilaments, or some combination of the two effects. Because cytochalasin D (CD) seems to have only one effect, the disruption of

microfilaments<sup>3-6</sup>, we used it here to study the role of microfilament organisation in the production of Moloney murine leukaemia virus (MLV). We report that actin and MLV structural proteins co-migrate in infected cells after CD treatment.

We found that CD ( $10^{-5}$  M) inhibited MLV production by chronically infected mouse thymus-bone marrow (TB) cells<sup>7</sup> by 70-80% (D.G.B. and G.Y.M., in preparation). To investigate the nature of this inhibition, untreated and CD-treated cells were stained for actin and for viral structural proteins by immunofluorescence. Anti-actin staining revealed a diffuse pattern of actin in the cytoplasm of both uninfected (Fig. 1a) and MLV-infected (Fig. 1b) untreated cells.

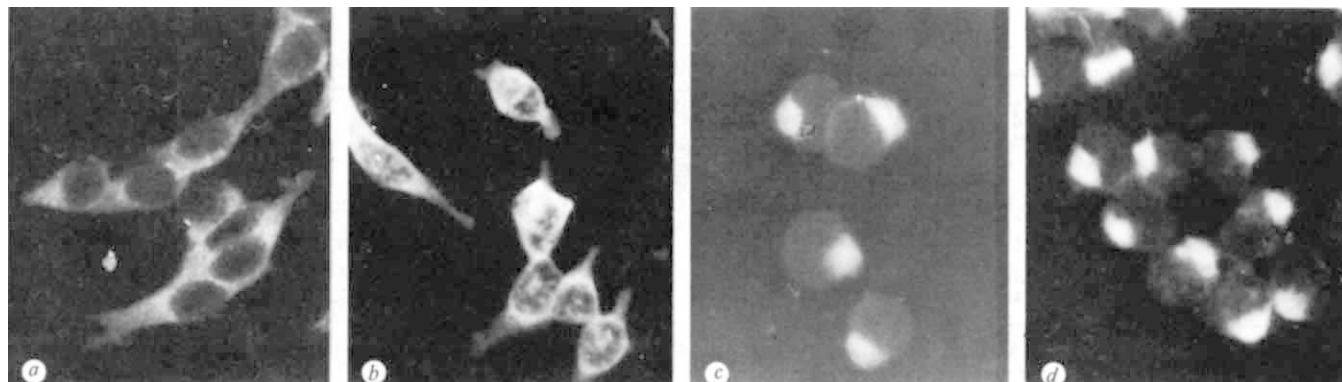
After CD treatment both uninfected (Fig. 1c) and MLV-infected (Fig. 1d) cells showed a cytoplasmic bulge which was capped with actin. This observation is consistent with the results reported by Sundqvist and Ehrnst<sup>8</sup>, who illustrated the polarisation of actin into a bulge of the cytoplasm following CB treatment. When these cells were stained for MLV proteins using a rabbit antiserum raised against detergent-disrupted whole virus, only the infected cells stained positively.

Co-capping of MLV proteins and actin was demonstrated in the same cell by double immunofluorescent staining using fluorescein-conjugated antibodies to localise actin, and rhodamine-conjugated antibodies to localise MLV proteins. Staining for both actin (Fig. 2a) and MLV proteins (Fig. 2b) occurred in a diffuse pattern in the untreated infected cells. In the CD-treated infected cells the immunofluorescent staining for both actin and MLV proteins was concentrated in the same cytoplasmic bulge of the cell. When uninfected cells, both CD-treated and untreated, were double stained for actin and MLV proteins, only the specific fluorescence for actin could be seen.

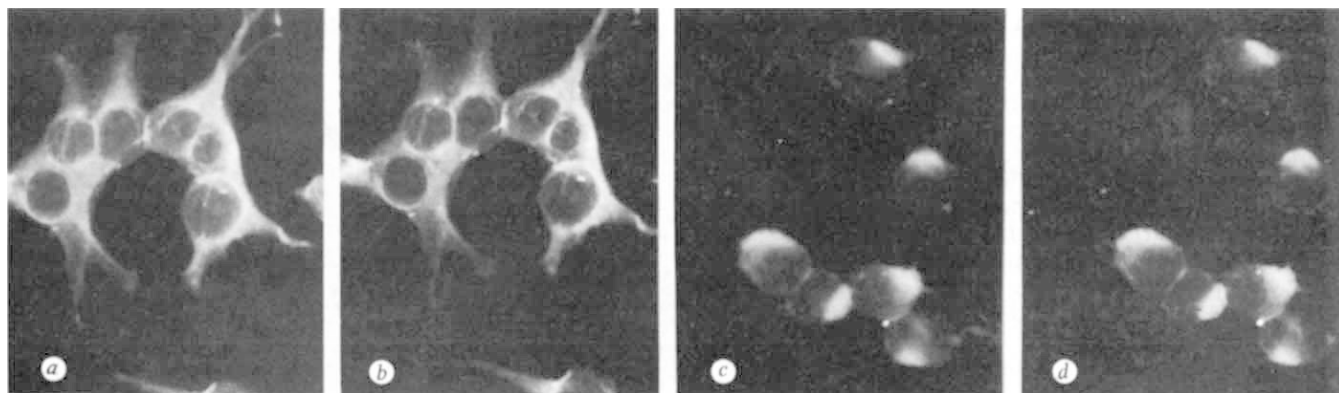
Actin has been shown to co-cap with cell surface antigens<sup>8</sup>, con A receptors<sup>9</sup> and immunoglobulins<sup>10</sup>. This phenomenon was considered an indication of a connection between actin and other cell surface proteins<sup>8-10</sup>. Koch and Smith<sup>11</sup> and Flanagan and Koch<sup>12</sup> have provided biochemical evidence for the association between actin and the major histocompatibility antigen H-2, and actin and surface immunoglobulin, respectively. On the other hand, actin has been reported to occur in enveloped viruses<sup>13</sup> and in murine mammary tumour viruses, where it may be involved in virus budding<sup>14</sup>. Our observations are consistent with these reports and demonstrate that actin and MLV structural proteins migrate into the CD-induced cytoplasmic bulge.

This work was supported by the MRC (G.Y.M. and J.R.T.) and NCI of Canada (J.B. and D.G.B.). We thank Drs S. Dales,

**Fig. 1** Immunofluorescent staining for actin. TB cells were infected in suspension as described by Ball *et al.*<sup>7</sup> and plated on tissue culture chamber slides (Lab-Tek). Cells were treated with  $10^{-5}$  M CD (Aldrich) which was initially dissolved in pure dimethyl sulphoxide and then diluted in normal media. After a 1 h treatment with CD, the cells were fixed for 5 min in absolute acetone at  $-20^{\circ}\text{C}$ , incubated with human anti-actin serum diluted 1:10 in 0.1 M phosphate-buffered saline (PBS), pH 7.4 for 30 min at room temperature, washed and further incubated with fluorescein isothiocyanate-anti-human IgG for 30 min. Untreated, control cells were processed in parallel. Anti-actin serum was obtained from a patient with active chronic hepatitis and the specificity of this serum for actin had been demonstrated previously<sup>15</sup>. Anti-actin adsorbed with actin was used as a control for staining as described previously<sup>15</sup>. a, Untreated, uninfected TB cells; b, untreated, MLV-infected TB cells; c, CD-treated, uninfected TB cells; d, CD-treated, MLV-infected TB cells. Magnification  $\times 1,000$ .







**Fig. 2** Double immunofluorescent staining for actin and MLV proteins. TB cells were treated and fixed as described in the legend to Fig. 1. Cells were stained for actin as described above, then stained for MLV proteins by incubating with rabbit anti-MLV serum diluted 1:40 in PBS for 30 min. Subsequently, the cells were washed and further incubated with rhodamine-labelled anti-rabbit IgG for 30 min. Normal rabbit serum which had been adsorbed with acetone-dried TB cells as described below, was used as a control. The cells were photographed using a Zeiss ultraviolet photomicroscope equipped with epi-illumination and specific filters for fluorescein and rhodamine. The anti-MLV serum was obtained from rabbits immunised with gradient-purified, detergent-disrupted MLV which had been grown on rat cells. It was adsorbed exhaustively with acetone-dried TB cells to remove nonspecific cellular antibodies. The serum showed broad spectrum reactivity and immunoprecipitating activity against the major viral structural components. *a*, Untreated, MLV-infected cells observed with fluorescein-specific filters; *b*, same cells as in (*a*) observed with rhodamine-specific filters; *c*, CD-treated, MLV-infected cells observed with fluorescein-specific filters; *d*, same cells as in *c* observed with rhodamine-specific filters.

J. A. McCarter, B. D. Sanwal and M. O. Creighton for critically reading the manuscript, Ann Finch for technical assistance, and Dr A. C. Wallace for human anti-actin serum.

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Received 13 February; accepted 3 July 1978.

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## Induction of specific antibodies to poly(ADP-ribose) in rabbits by double-stranded RNA, poly(A) · poly(U)

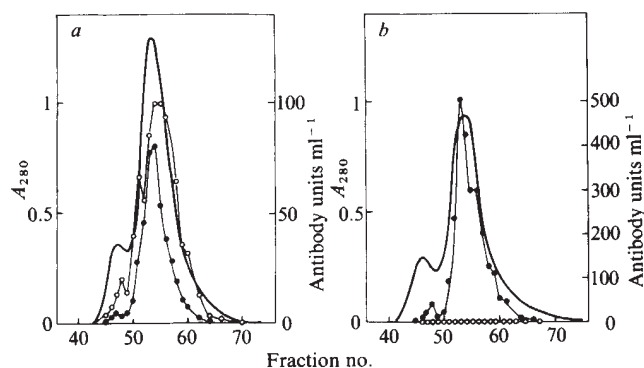
WE have reported<sup>1</sup> the presence of naturally occurring antibodies to poly(ADP-ribose) in the sera of patients with systemic lupus erythematosus (SLE). During our study we discovered a population of antibodies cross-reacting with poly(A) · poly(U) among the antibodies to poly(ADP-ribose) and found that this population could be completely removed by absorption of the SLE sera with poly(A) · poly(U)<sup>1</sup>. The mechanism of the cross-reaction to poly(A) · poly(U) of some antibodies to poly(ADP-ribose) is unknown. Poly(ADP-ribose)

is a biopolymer which is synthesised by nuclear enzyme(s)<sup>2</sup>. Recent advance in the study of this biopolymer has been reviewed<sup>3,4</sup>. We have found<sup>5</sup> that poly(ADP-ribose) shows strong antigenicity in rabbits and that rabbit antibodies to poly(ADP-ribose) do not cross-react at all with synthetic homopolynucleotides. However, the cross-reactions of rabbit antibodies with double-stranded RNA, poly(A) · poly(U) or poly(I) · poly(C), have not been fully elucidated. Therefore, we tested whether poly(A) · poly(U) can induce specific antibodies to poly(ADP-ribose) and, conversely, whether poly(ADP-ribose) can induce antibodies cross-reacting with poly(A) · poly(U). We describe here that induction of specific antibodies to poly(ADP-ribose) in rabbits could be achieved by injecting a complex of poly(A) · poly(U) and methylated bovine serum albumin (MBSA) in Freund's complete adjuvant (FCA); moreover, poly(A) · poly(U) has a specific effect in inducing antibodies to poly(ADP-ribose), as neither poly(I) · poly(C) nor poly(A) induce antibodies to poly(ADP-ribose).

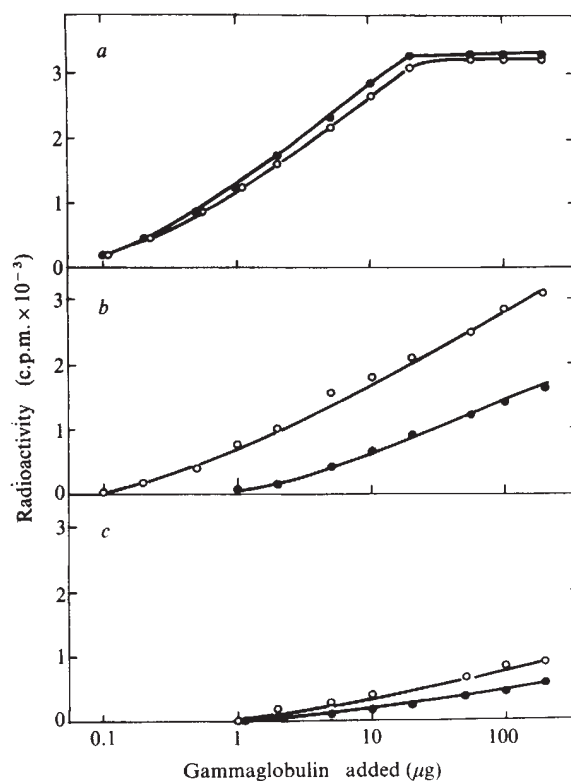
Figure 1a shows the profiles of antibody titres to poly(A) · poly(U) and poly(ADP-ribose) in the fractions eluted from a column of Sephadex G-150, on to which rabbit gammaglobulin (2 ml), obtained from a rabbit immunised with poly(A) · poly(U)-MBSA complexes in FCA, had been applied. Antibody activities to both poly(A) · poly(U) and poly(ADP-ribose) were observed in the 19S and 7S gammaglobulin fractions, although those in the latter fraction were much higher. No antibody activity to poly(A) · poly(U) was detected in fractions from a Sephadex column with adsorbed gammaglobulin (2 ml) obtained from a rabbit immunised with poly(ADP-ribose)-MBSA complexes in FCA, even though, as shown in Fig. 1b, the specific activity of the antibodies to poly(ADP-ribose) in the 7S peak fraction (expressed as antibody units per 1  $A_{280}$  unit) was approximately sevenfold higher than that of Fig. 1a. Similar results were obtained in paired experiments carried out to confirm the data shown in Fig. 1.

Figure 2 shows <sup>14</sup>C-poly(ADP-ribose) binding to gammaglobulin fractions obtained from rabbits immunised with poly(A) · poly(U)-MBSA in FCA, or poly(ADP-ribose)-MBSA in FCA and from an untreated rabbit, respectively, as a function of the gammaglobulin concentration. <sup>14</sup>C-poly(ADP-ribose) binding to gammaglobulin fractions was measured before and after absorption of the gammaglobulin with poly(A) · poly(U). As shown in Fig. 2b, the remaining antibody activity was found to be approximately half the original with high doses of gammaglobulin, in the case of a rabbit immunised with poly(A) · poly(U)-MBSA in FCA. However, it took about

ten times as much globulin, judged from Fig. 2b more accurately to reach a given level of binding after absorption. This could be interpreted as indicating that about 10% of the original antibody activity was left. The antibody activity of the gammaglobulin from a rabbit immunised with poly(ADP-ribose)-MBSA in FCA was practically unchanged at all concentrations of gammaglobulin even after absorption (Fig. 2a). As shown in Fig. 2c, a little  $^{14}\text{C}$ -poly(ADP-ribose) was observed in the gammaglobulin fractions from the untreated rabbit, half of the original binding activity still remaining after absorption with poly(A)·poly(U). Further study is required to determine whether this slight remaining binding activity is actually due to antibodies to poly(ADP-ribose).  $^3\text{H}$ -poly(A)·poly(U) binding activity of the gammaglobulin absorbed with poly(A)·poly(U)-MBSA complex was actually reduced to that of the gammaglobulin obtained from an untreated rabbit (Fig. 3). Thus, it was shown that poly(A)·poly(U)-MBSA complex used as an immunoabsorbent was effective in removing almost all the antibodies that react with poly(A)·poly(U). This was further confirmed using IgG fraction purified from the absorbed gammaglobulin by a DE52 cellulose column.



**Fig. 1** New Zealand White rabbits (female, 2.5–3 kg body weight) were used throughout. Two rabbits *a* and *b* were used for each antigen. Antibodies to poly(ADP-ribose) were prepared as described previously<sup>5</sup>. The procedure for immunisation with poly(A)·poly(U) was essentially the same as that with poly(ADP-ribose), except for the doses of antigen.  $^{14}\text{C}$ -poly(ADP-ribose) and unlabelled poly(ADP-ribose) were prepared by the method of Sugimura *et al.*<sup>6</sup>. Poly(A)·poly(U) duplex was prepared by mixing equimolar solutions of poly(A) and poly(U) (Miles).  $^3\text{H}$ -poly(A)·poly(U) was prepared as follows:  $^3\text{H}$ -poly(A) (3.15 Ci mmol<sup>-1</sup> AMP; New England Nuclear) was adjusted to  $1.15 \times 10^5$  c.p.m. per  $\mu\text{g}$  poly(A) by adding unlabelled poly(A).  $^3\text{H}$ -poly(A)·poly(U) ( $3.4 \times 10^3$  c.p.m. per 50 ng) duplex was prepared as described above. One mg of poly(A)·poly(U) and 1 mg MBSA both in 0.5 ml distilled water were mixed and emulsified in FCA (Difco) (1 ml). Then 2 mg of the antigen was injected per rabbit. One month after injection of poly(ADP-ribose) or poly(A)·poly(U), blood was collected by heart puncture. Each serum was then inactivated by heating at 56 °C for 30 min and the gammaglobulin was isolated as described previously<sup>1</sup>. Gammaglobulin (2 ml) was applied to a column of Sephadex G-150 (1.8 × 84 cm), which had been equilibrated with PBS, pH 7.4.  $^{14}\text{C}$ -poly(ADP-ribose) binding to gammaglobulin fractions was assayed by the Millipore filter method as described previously<sup>1</sup>, but a standard curve for the quantitative determination of antibody was constructed, as in experiments on mice<sup>7</sup>, using rabbit antiserum to poly(ADP-ribose); one unit of antibody was defined as the amount giving 50% of the maximum bound radioactivity. Thus, antibody could be expressed as units per ml of fraction.  $^3\text{H}$ -poly(A)·poly(U) binding was assayed by essentially the same methods as poly(ADP-ribose), except that the mixture was incubated at 0 °C for 2 h, because poly(A)·poly(U) was slightly degraded by contaminating RNase during incubation at 37 °C. Antibody was determined using a standard curve obtained with a rabbit immunised with poly(A)·poly(U), as in the case of poly(ADP-ribose). Antibody was again expressed as units per ml of fraction. —●—, antibody to poly(ADP-ribose); —○—, antibody to poly(A)·poly(U).  $A_{280}$ , —.



**Fig. 2** Five mg of poly(A)·poly(U) in distilled water and 5 mg MBSA were mixed and the complex formed was washed four times with PBS at 4 °C. The precipitate was suspended in 2 ml PBS. To this suspension was added 10 mg gammaglobulin dissolved in 2 ml PBS, obtained from rabbits immunised as described in the text. The suspension prepared by mixing 5 mg poly(A)·poly(U) and MBSA was used for the absorption experiment. A mixture of MBSA-poly(A)·poly(U) suspension and gammaglobulin (10 mg) was occasionally shaken for 1 h at 0 °C, kept at 0 °C overnight, and then centrifuged at 105,000 g for 1 h at 4 °C. The supernatant was used as absorbed gammaglobulin for the  $^{14}\text{C}$ -poly(ADP-ribose) binding assay.  $^{14}\text{C}$ -poly(ADP-ribose) binding to gammaglobulin with or without absorption was carried out as described in Fig. 1. *a*, Gammaglobulin obtained from a rabbit immunised with poly(ADP-ribose)-MBSA in FCA; *b*, gammaglobulin from a rabbit immunised with poly(A)·poly(U)-MBSA in FCA; *c*, gammaglobulin from an untreated rabbit. ○, Before absorption; ●, after absorption.

It is very important to confirm that the remaining  $^{14}\text{C}$ -poly(ADP-ribose) binding after absorption of the gammaglobulin fractions obtained from rabbits immunised with poly(A)·poly(U)-MBSA in FCA (Fig. 2b) is specific for poly(ADP-ribose). Table 1 shows the specificity of binding of  $^{14}\text{C}$ -poly(ADP-ribose) to the respective absorbed gammaglobulin fractions from two rabbits (A and B), immunised with poly(A)·poly(U)-MBSA in FCA. The specificity of antibodies to poly(ADP-ribose) obtained from a rabbit immunised with poly(ADP-ribose)-MBSA in FCA is also shown in Table 1 (rabbit C). There is no apparent difference in the binding specificities of the gammaglobulins obtained from rabbits immunised with poly(A)·poly(U) and poly(ADP-ribose), in the inhibition shown in Table 1.

To determine whether the specific  $^{14}\text{C}$ -poly(ADP-ribose) binding is attributable to antibody molecules, the IgG fraction of gammaglobulin from a rabbit immunised with poly(A)·poly(U)-MBSA in FCA was separated by DE52 cellulose (Whatman) column chromatography.  $^{14}\text{C}$ -poly(ADP-ribose) binding activity was recovered in the IgG fraction, and  $\text{F(ab')}_2$ , isolated from IgG by the procedure of Nisonoff *et al.*<sup>8,9</sup>, retained most of the activity (data not shown), as in the case of SLE sera<sup>1</sup>. However,  $^{14}\text{C}$ -poly(ADP-ribose) binding activity was very low in  $\text{F(ab')}_2$  from an untreated rabbit (data not



shown). Thus, it is concluded that poly(A)·poly(U)-MBSA in FCA induces specific antibodies to poly(ADP-ribose).

Poly(A)·poly(U) is known to have adjuvant activity by itself<sup>10-12</sup>, and thus the effect of poly(A)·poly(U) in inducing antibodies to poly(ADP-ribose) may be due to polyclonal activations of antibody forming cells. However, this possibility can be excluded by the following findings: a complex of poly(I)·poly(C)-MBSA or poly(A)-MBSA with FCA did not induce antibodies to poly(ADP-ribose) (data not shown); poly(I)·poly(C) has as strong adjuvant activity as poly(A)·poly(U)<sup>13,14</sup>; and poly(A)·poly(U) did not induce antibodies to double-stranded DNA or to sheep red blood cells (data not shown).

Our experiment has shown that about 10% of the original <sup>14</sup>C-poly(ADP-ribose) binding of antibody molecules remained, even after absorption with poly(A)·poly(U), and that this was not inhibited by poly(A)·poly(U). Furthermore, the activities of antibodies obtained from rabbits immunised with poly(ADP-ribose)-MBSA complexes were essentially the same before and after absorption with poly(A)·poly(U). Thus, it is unlikely that antibody induction was due to a structural similarity between poly(ADP-ribose) and poly(A)·poly(U), that is, the presence of common antigenic determinants. However, there is as yet no direct evidence to support this possibility. Therefore, the antibody population that reacts with poly(ADP-ribose) other than the immunising antigen poly(A)·poly(U) may be 'heteroclitic'<sup>15-17</sup>.

In our experiment, the poly(A)·poly(U) used for both immunisation and absorption was prepared in distilled water and mixed with MBSA in water. The double-helical poly(A)·poly(U) structure would not be as stable in distilled water as in physiological saline. Its melting point would be lower than that of poly(A)·poly(U) formed in physiological saline. Therefore some free poly(A) that may be present in distilled water could exist as a single-stranded helix, structurally resembling poly(ADP-ribose). Thus, antibodies induced by it would

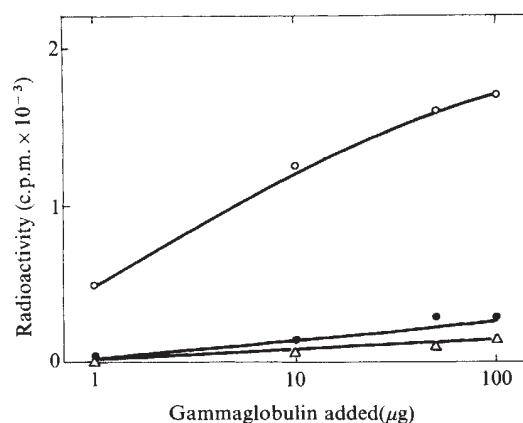


Fig. 3 See Figs 1 and 2 for details of <sup>3</sup>H-poly(A)·poly(U) binding assay and the absorption experiment. ○, Before absorption; ●, after absorption; Δ, gamaglobulin from an untreated rabbit.

react with poly(ADP-ribose) but not with poly(A)·poly(U) in PBS, which was used in the <sup>14</sup>C-poly(ADP-ribose) binding assay. To determine whether or not poly(A)·poly(U) prepared in PBS would also induce the antibody population that reacts specifically with poly(ADP-ribose), poly(A)·poly(U)-MBSA complex prepared in PBS was injected into two New Zealand White rabbits as described in Fig. 1. The results obtained were essentially the same as in the case of poly(A)·poly(U) prepared in distilled water. We suggest, therefore, that the residual <sup>14</sup>C-poly(ADP-ribose) binding after absorption was due to induction of antibody-forming cells against poly(ADP-ribose) by some unknown mechanism. Whether or not the antibody population that specifically reacts with poly(ADP-ribose) is due to 'heteroclitic' remains as an interesting problem to be studied.

Our data also suggest that poly(A)·poly(U) or structurally related double-stranded RNA other than poly(I)·poly(C) may have some role in the production of antibodies to poly(ADP-ribose) in SLE patients. It has been reported<sup>18</sup> that heterogeneous nuclear RNA purified from HeLa cells has inter-molecular duplexes with 300 base pairs within about 7,000 nucleotides. Furthermore, cytoplasmic filamentous structures resembling unenveloped nucleocapsids of paramyxo- or related viruses were found in the tissues from SLE patients<sup>19</sup>. Double-stranded RNA is found in tissues infected with RNA viruses, especially during the replicative stages of virus infection<sup>20</sup>. Thus, as no clear correlations between antibody titres to poly(ADP-ribose) and to poly(A)·poly(U) could be observed in 20 SLE patients examined (unpublished data), studies must be made for possible immune complexes formed between poly(ADP-ribose) and its antibodies in the blood of SLE patients.

This work was supported by grants from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare, Japan.

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Received 13 December 1977; accepted 6 July 1978.

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Table 1 Inhibition of <sup>14</sup>C-poly(ADP-ribose) binding to the gamaglobulin fraction by unlabelled poly(ADP-ribose) and related compounds

| Related compound            | Rabbit<br>gamaglobulin<br>% Inhibition |    |     |
|-----------------------------|----------------------------------------|----|-----|
|                             | A                                      | B  | C   |
| Poly(ADP-ribose)            | 91                                     | 89 | 100 |
| Poly(A)·poly(U)             | 16                                     | 0  | 11  |
| Poly(I)·poly(C)             | 14                                     | 0  | 5   |
| Poly(A)                     | 7                                      | 15 | 0   |
| Poly(U)                     | 15                                     | 5  | 0   |
| Poly(I)                     | 6                                      | 0  | 5   |
| ADP-ribose                  | 7                                      | 8  | 0   |
| Calf thymus DNA (native)    | 0                                      | 9  | 5   |
| Calf thymus DNA (denatured) | 0                                      | 8  | 0   |
| SV40 viral DNA              | 0                                      | 0  | 0   |
| Yeast RNA                   | 7                                      | 0  | 0   |
| <i>E. coli</i> t RNA        | 11                                     | —* | 6   |
| Rat liver ribosomal RNA     | 0                                      | 0  | 0   |
| 5'-AMP                      | 6                                      | 0  | 2   |
| 3'-AMP                      | —                                      | 0  | 1   |

The inhibition experiment was carried out essentially by the method<sup>1</sup> used in cases of SLE. In the absence of unlabelled poly(ADP-ribose) or related compounds, samples from rabbits A, B and C were adjusted to bind 50, 55 and 65% of the radioactivity added as <sup>14</sup>C-poly(ADP-ribose), respectively—that is, the amounts of gamaglobulins were 200, 400 and 2.5 μg, respectively. A and B, rabbits immunised with poly(A)·poly(U)-MBSA in FCA. The respective gamaglobulin fractions from rabbits A and B were absorbed with poly(A)·poly(U)-MBSA complexes, as described in Fig. 2; C, rabbit immunised with poly(ADP-ribose)-MBSA in FCA, the gamaglobulin not absorbed with poly(A)·poly(U)-MBSA complexes.

\* Not tested.

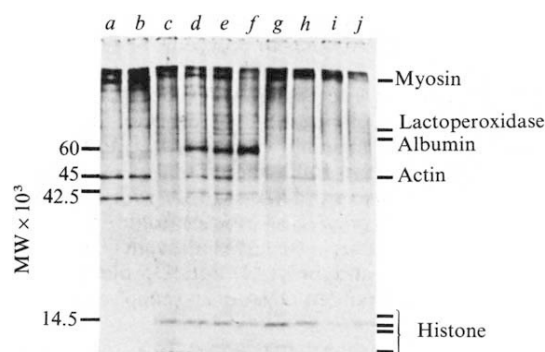
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## Transformation-specific antigen induced by oncogenic human adenovirus

A TEMPERATURE-SENSITIVE (*ts*) mutant of the highly oncogenic group A (ref. 1) human adenovirus type 12 (H12), H12*ts*401 (ref. 2) is unable to establish stable transformation of cells in restrictive conditions<sup>3</sup>. Cells transformed by *ts*401 at the permissive temperature and shifted to nonpermissive temperatures show a reversion to a normal phenotype; wild-type H12-transformed cells, in contrast, exhibit a transformed phenotype when grown in either restrictive or permissive conditions<sup>4</sup>. The temperature sensitivity of the transformed phenotype of the *ts*401 mutant-transformed cells suggests that the continued expression of gene 401 is required for maintenance of the transformed cell phenotype. This study was initiated to detect the H12 transformation-specific protein(s) in H12-transformed cell lines. We identify here a 60,000 molecular weight transformation-specific antigen in H12-transformed rat 3Y1 cells<sup>5,6</sup> by immunoprecipitation of <sup>35</sup>S-methionine labelled polypeptides with serum from H12 tumour-bearing hamsters. Furthermore, the expression of this antigen is temperature dependent in 3Y1 cells transformed by *ts*401. Further characterisation of the 60,000 MW antigen may lead to an understanding of the molecular mechanism of adenovirus cell transformation.

Cloned lines of H12 wild type (WT)-transformed 3Y1 cells (3Y1/WT-10)<sup>4</sup>, *ts*401 transformed (3Y1/401-4)<sup>4</sup> and untransformed 3Y1 cells were grown and labelled with <sup>35</sup>S-methionine at 35 °C and 40 °C. Extracts of the radiolabelled cells were reacted with serum from hamsters bearing tumours induced by hamster embryo cells transformed *in vitro* by H12. The tumour-specific (T) H12 antigen(s) was isolated using the staphylococcal protein A-antibody adsorbent technique<sup>7</sup>, and analysed by SDS-polyacrylamide gel electrophoresis (Fig. 1). One predominant polypeptide with an apparent MW of 60,000 was immunoprecipitated by anti-T serum from cells transformed by WT and grown at 35 °C (lane f) or 40.5 °C (lane e). The striking finding is that this component was absent in extracts from cells transformed by *ts*401 and grown at 40.5 °C (lane c), but was present in these cells grown at 35 °C (lane d). Moreover, this antigen was not present in extracts from untransformed 3Y1 cells grown at 35 °C (or at 40.5 °C, data not shown) and treated with anti-T (lane a) or normal (pre-immune) serum (lane b), or in any of the transformed cell extracts immunoprecipitated with normal serum (lanes g–j).

Another relatively prominent component in transformed cell extracts was a polypeptide with an apparent MW of 14,500, but this polypeptide was immunoprecipitated with normal (Fig. 1, lanes g–j) or anti-T serum (Fig. 1, lanes c–f). Additional minor polypeptides, some with MWs of 42,500 and 45,000, were observed in transformed cell extracts treated with anti-T serum (Fig. 1, lanes c–f), but all of these could be detected as well with normal serum (Fig. 1, lanes g–j). The 60,000 MW antigen, therefore, seemed to be the only component that was immunoprecipitated by anti-T and not by normal serum, and that was strongly reduced in *ts*401-transformed cells in restric-



**Fig. 1** SDS-polyacrylamide gel analysis of polypeptides immunoprecipitated from <sup>35</sup>S-methionine-labelled untransformed and H12WT- and H12*ts*401-transformed 3Y1 cells. Two to four 100-mm Petri dishes were seeded each with 10<sup>7</sup> cells from subconfluent cultures passed two or three times at 35 °C or 40.5 °C. One day later, the cultures were labelled for 3 h at the appropriate temperatures with 22 µCi ml<sup>-1</sup> <sup>35</sup>S-methionine (Amersham/Searle, 1,155 Ci mmol<sup>-1</sup>) in Eagle's medium lacking methionine and supplemented with 2% dialysed fetal bovine serum. The cells were washed three times with a buffered salt solution containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA, and then lysed in RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris-HCl, pH 7.2)<sup>21</sup> containing 0.3 mg ml<sup>-1</sup> phenylmethylsulphonyl fluoride, a protease inhibitor. The cell lysates were sonicated for 2 min at full power in a Raytheon DF-101 sonic oscillator. The sonicates were clarified at 12,000g for 15 min. The protein A antibody adsorbent technique<sup>7</sup> was used for isolating the T antigen from the supernatants. To reduce the background, the cell extracts were first treated with undiluted normal (pre-immunisation) hamster serum for 18 h at 0 °C at a ratio of 50 µl of serum per 150 µl of extract. The IgG molecules of this serum were removed by adding 200 µl of a 10% suspension of the protein A-containing bacterium, *Staphylococcus aureus*, strain Cowan I (ref. 7) to the mixture for 10 min at room temperature. The bacteria were removed by centrifugation at 2000g for 20 min. 150 µl aliquots of supernatant were incubated with 100 µl of the indicated serum containing 1 mg of serum protein. After 30 min at room temperature, 200 µl of the above bacteria were added for 10 min at room temperature to adsorb the immune complexes. The bacteria were pelleted and washed three times with RIPA buffer, suspended in electrophoresis sample buffer containing 2% SDS, 15% (v/v) glycerol, 1% 2-mercaptoethanol, 0.075 M Tris-sulphate, pH 8.4, and 0.001% bromophenol blue, heated at 100 °C for 10 min, and pelleted. The supernatant was electrophoresed through a discontinuous SDS-polyacrylamide slab gel system<sup>22,23</sup>. The stacking gel contained 5% acrylamide, 0.12% bisacrylamide, and 0.075 M Tris-sulphate, pH 8.4; the 20% separating gel contained 20% acrylamide, 0.1% bisacrylamide, and 0.375 M Tris-sulphate, pH 8.4. The 0.065 M Tris-borate, pH 8.4, tank buffer contained 0.1% SDS. Approximately 5,000 c.p.m. of radioactive protein was loaded per well. Electrophoresis was performed at room temperature for 6–7 h at a constant current of 40 mA per gel. After electrophoresis, gels were treated for fluorography<sup>24</sup>. Dried gels were exposed to Kodak Royal RP X-omat X-ray film; films were preflashed to provide a linear response<sup>25</sup>. MW markers used were: myosin (200,000), lactoperoxidase (73,000), bovine serum albumin (65,000), actin (45,000) and calf histones (11, 13, 15, 16). Anti-tumour (anti-12 T) serum was obtained from hamsters bearing tumours induced by hamster embryo cells transformed *in vitro* by adenovirus type 12. Normal (pre-immune) and immune sera were obtained from Dr Bruce Casto (BioLabs). Lanes a, b display polypeptides from untransformed 3Y1 cells grown at 35 °C immunoprecipitated with: a, anti-12 T serum; b, normal serum. c–f, Polypeptides immunoprecipitated with anti-12 T serum from: c, *ts*401-transformed cells grown at 40.5 °C; d, *ts*401-transformed cells grown at 35 °C; e, WT-transformed cells grown at 40.5 °C; f, WT-transformed cells grown at 35 °C. g–j, Polypeptides immunoprecipitated with normal serum from: g, *ts*401-transformed cells grown at 40.5 °C; h, *ts*401-transformed cells grown at 35 °C; i, WT-transformed cells grown at 40.5 °C; and j, WT-transformed cells grown at 35 °C.



tive conditions (Fig. 1). Similar results were obtained with another independently isolated ts401-transformed 3Y1 cell line (3Y1/401-2) (data not shown). Of particular interest is the finding that only one major protein band of 58,000 MW is detected in two different adenovirus group C (ref. 1) transformed cell lines when sera from animals bearing adenovirus group C induced tumours were used; this antigen may be the adenovirus gene product common to cells transformed by this group of viruses<sup>8</sup>. The group C adenoviruses are non-oncogenic but they transform cultured rodent cells<sup>1</sup>. Although the transforming gene sequence of group A and C adenoviruses is different<sup>9</sup>, the transforming gene of members of both groups is located on the left end (7–8%) of the viral genome<sup>10–12</sup>.

The H12 tumour-specific antigen(s) represents a subset of the proteins synthesised early in lytically infected cells<sup>14–17</sup>. A 60,000 MW protein has been specifically immunoprecipitated by H12 tumour serum from H12 early-infected KB cells<sup>17</sup>. In these cells several smaller proteins ranging in size from 10,000 to 16,500 also have T-antigen specificity<sup>17</sup>. More recently, several proteins have been identified in H12 early-infected KB cells by immunoprecipitation using antisera prepared in rats against H12-induced proteins synthesised in rat cells transformed by the H12 EcoRI-C fragment, which is located at the left end of the physical map between 0 to 16 units<sup>18</sup>. Four polypeptides of apparent MW 40,000 to 46,000 as well as 10,500 and 15,500 were immunoprecipitated (M. Green, personal communication). There was no clear indication, however, that a common polypeptide was precipitated by the several antisera tested. The 40,000 to 46,000 polypeptides may be subspecies of the 60,000 polypeptide.

Considerable purification of the H12 T antigen(s) has been achieved by taking advantage of the strong binding affinity of this antigen for double-stranded DNA<sup>19,20</sup>. The T-antigen polypeptides were immunoprecipitated from the purified preparations by serum from H12 tumour-bearing hamsters and were identified as a major peptide of approximate MW 50,000 and a minor peptide of MW 11,000<sup>13</sup>. If the 60,000 MW protein detected in our study is indeed the gene product common to H12-transformed cells, it must be coded for, or induced by, the segment of the left-hand end of the genome within map positions 0 to 7.2, since cells have been transformed by the H12 EndoR·HindIII-G fragment<sup>12</sup>. 3Y1 cells transformed by the HindIII-G fragment of the H12 genome contain T antigen(s)<sup>12</sup>, and it is likely that this antigen is coded by at least a portion of this region of the genome. A different rat cell line transformed by the whole viral DNA of H12 has been found to contain both T antigen and another early H12-induced protein, a single-strand specific DNA binding protein with apparent MW 60,000<sup>12,16</sup>. As the single-strand specific DNA binding protein is not detectable in all H12 transformed cells<sup>12</sup>, it clearly is not essential for maintenance of cell transformation. The 60,000 MW protein detected in our study, on the other hand, is the only major protein immunoprecipitable from each of several different H12-transformed cell lines when serum from animals bearing H12-induced tumours is used. Moreover, this protein is transformation dependent in that it is not detectable in mutant transformed cells under restrictive conditions, where the cells lose at least part of their transformed phenotype. The altered behaviour of the 60,000 MW protein in mutant infection strongly suggests that it is the product of the 401 gene. Alternatively, this protein may be a cell-coded protein, induced by the 401 function, and its reduction in restrictive conditions may reflect host cell alterations associated with a return to the normal phenotype.

We thank Dr Lloyd A. Culp for advice and aid with slab gel electrophoresis, and Dr G. Kimura for 3Y1 cells. This work was supported by the NIH (National Cancer Institute grant CA 13231) and The University of Akron (RG 495).

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Received 12 May; accepted 26 June 1978.

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## Galactocerebroside is a specific cell-surface antigenic marker for oligodendrocytes in culture

THE increasing use of tissue culture techniques in neurobiological studies has created an urgent need for cell-type specific markers which would allow unambiguous identification of the different types of neural cells. We show here that galactocerebroside (GC), the major glycolipid in myelin<sup>1</sup>, can serve as such a cell-surface marker for cultured rat oligodendrocytes, the glial cells responsible for making myelin in the central nervous system.

Two different rabbit anti-galactocerebroside (R anti-GC) antisera were used and gave similar results. One was produced by four weekly inoculations, each consisting of 1 mg of bovine brain cerebroside (99% GC, Sigma) and 5 mg egg albumin (Sigma) in 0.25 ml of saline, emulsified with an equal volume of complete Freund's adjuvant (CFA, Difco)<sup>2</sup>. The rabbit was bled 4 weeks after the final inoculation. The other antiserum was produced by a single immunisation with 2.5 mg of purified human brain GC and 2.5 mg of bovine serum albumin (BSA, Sigma) in saline, emulsified with an equal volume of CFA<sup>3</sup>. The human brain GC was purified by the method of Kishimoto *et al.*<sup>4</sup>, and its purity determined by thin-layer chromatography using chloroform-methanol-water (70:30:5) or borate-impregnated plates to check for the presence of glucocerebroside<sup>5</sup>; in both cases, only the double spot of GC was seen. The rabbit was bled after 2 weeks. Both sera were heated for 30 min at 56°C, and the first serum was adsorbed with 10% (v/v) bovine liver homogenate. When assayed by radioimmunoprecipitation<sup>2,6</sup>, the first serum had an anti-GC titre of 1:530, and the second had a titre of 1:2,000 as determined by complement fixation<sup>3</sup>. All of the staining activity of both sera in indirect immunofluorescence tests (see below) was removed by two adsorptions with purified rat central myelin (50 mg wet weight per 100 µl serum for 1 h at 20°C), or by adsorption with a suspension of micelles<sup>7</sup> containing cholesterol, lecithin and GC, but not by similar suspensions devoid of glycolipid, or containing glucocerebroside (purified from the spleen of a patient with Gaucher's disease and checked for purity as above). Rabbit antisera raised as described against BSA were inactive in immunofluorescence assays.

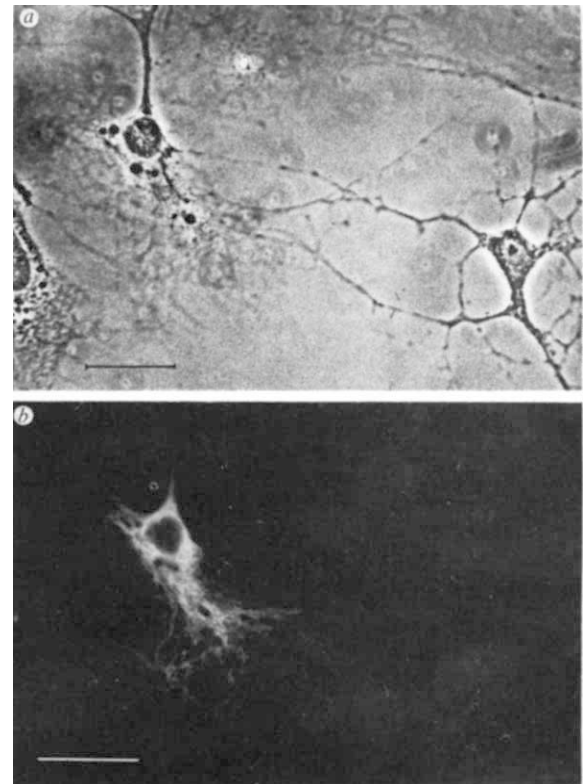
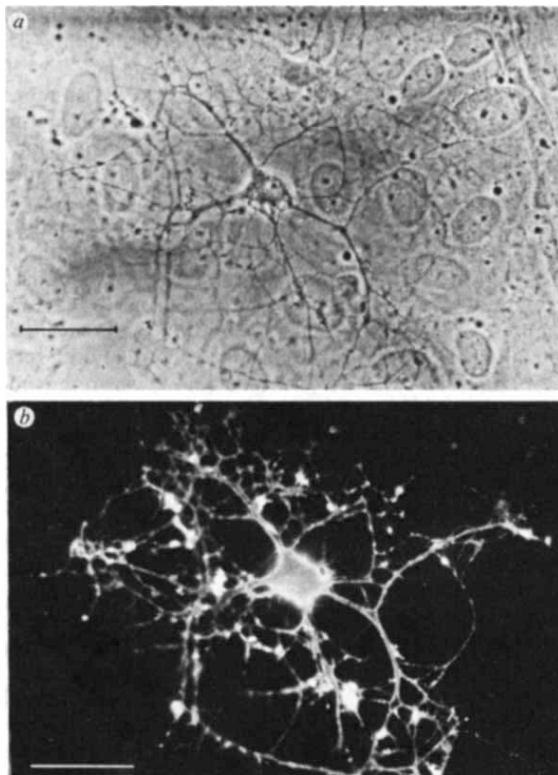
Optic nerves from 6–8-d-old Wistar/Furth rats were removed aseptically, dissociated in trypsin and collagenase, and the resulting cell suspension was plated on 13-mm glass coverslips (10,000–15,000 cells per coverslip), in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, in Linbro multiwell plates as described previously for culturing cells from newborn rat sciatic nerve<sup>8</sup>. After 3–6 days in culture, the cells on

coverslips were exposed to R anti-GC serum (diluted 1:20 or 1:50) for 25 min at 20 °C, washed and incubated in goat anti-rabbit IgG conjugated to rhodamine (G anti-RIg-Rd; Nordic, batch no. 5-874) diluted 1:50 for 25 min. After washing, the cells were fixed in 5% acetic acid in 95% ethanol (acid-alcohol) for 10 min at -20 °C, washed and mounted on glass slides for viewing in a Zeiss Universal fluorescence microscope equipped with phase contrast, fluorescein and rhodamine optics as described previously<sup>8</sup>.

There were two principal morphological cell types in these cultures: fibroblastoid cells, which were usually large and flat, and process-bearing cells, which were usually smaller and darker with extended processes which were often long and/or branched. In confluent cultures (usually >5 d old) the process-bearing cells tended to be on top of a monolayer of fibroblastoid cells (Fig. 1). In all cultures, more than 75% of the cells were fibroblastoid. In indirect immunofluorescence tests, approximately 5% of the cells ( $5 \pm 1.5$  in four experiments) were labelled intensely with R anti-GC serum, while the remaining cells were unlabelled (Fig. 1). All of the labelled cells were process-bearing; most of them had small, dark nuclei, dark cytoplasm and multiple, extensively branched processes. Most of the process-bearing cells, however, were unlabelled.

Optic nerves do not contain neuronal cell bodies; in rat optic nerve of this age, myelination has just begun and the majority of cells are oligodendrocytes and astrocytes and their precursors<sup>9,10</sup>. Thus, it seemed likely that the cells which labelled with the R anti-GC sera were either oligodendrocytes or fibrous astrocytes, or both. To distinguish between these possibilities we used a rabbit antiserum against the "glial fibrillary acidic protein"<sup>11,12</sup> (R anti-GFAP, provided by Drs D. Dahl and A. Bignami<sup>13</sup>), a protein associated with glial filaments<sup>11,14</sup> and which has been shown to be specific for astrocytes in

**Fig. 1** GC<sup>+</sup> cell (putative oligodendrocyte) on top of a monolayer of GC<sup>-</sup> fibroblast-like cells in dissociated cell culture of optic nerves from 6-d-old rats. After 6 d in culture, the cells were labelled with R-anti-GC serum (1:20) followed by G anti-RIg-Rd (1:50), fixed in acid-alcohol and then viewed with phase contrast (upper) or fluorescence (lower) optics. Scale bar, 10  $\mu$ m.



**Fig. 2** GFAP<sup>+</sup> cell (putative astrocyte) in optic nerve culture from 7-d-old rats. After 4 d in culture, the cells were fixed in acid-alcohol, labelled with R anti-GFAP serum (1:50) followed by G anti-RIg-Fl (1:100) and viewed with phase contrast (upper) or fluorescence (lower) optics. Note that the fibroblast-like cell (mostly out of the picture on the left) and the process-bearing cell (putative oligodendrocyte) on the right are GFAP<sup>-</sup>. Scale bar, 10  $\mu$ m.

immunofluorescence<sup>12</sup> and immunoperoxidase<sup>15</sup> studies on frozen sections of brain. When cultures of dissociated cells from optic nerves were fixed in acid-alcohol and exposed to R anti-GFAP (1:50) and G anti-RIg conjugated to fluorescein (G anti-RIg-Fl, 1:100; Nordic, batch no. 27-175), 10-20% of the cells ( $15 \pm 4$  in six experiments) were intensely labelled, and the remainder did not label at all. The labelled cells usually stained in a distinctive fibrillar pattern (Fig. 2) and included cells of both process-bearing (Fig. 3) and fibroblastoid (Fig. 4) morphology. These latter presumably correspond to protoplasmic astrocytes, which *in vivo* are found mainly in grey matter, and the former to fibrous astrocytes, which are found primarily in white matter. GFAP<sup>+</sup> cells with intermediate morphology (Fig. 2), however, were not infrequently seen in the cultures. Approximately 75% of process-bearing cells were GFAP<sup>+</sup>. The R anti-GFAP serum did not stain any cells in acid-alcohol fixed cultures of rat sciatic nerve, dorsal root ganglion, muscle or in frozen sections of sciatic nerve, which attests to the astrocyte-specificity of the serum in our immunofluorescence assay. No labelled cells were seen when living cells in unfixed cultures of dissociated optic nerves were exposed to R anti-GFAP serum, indicating that GFAP is not expressed in detectable amounts on the surface of cultured astrocytes.

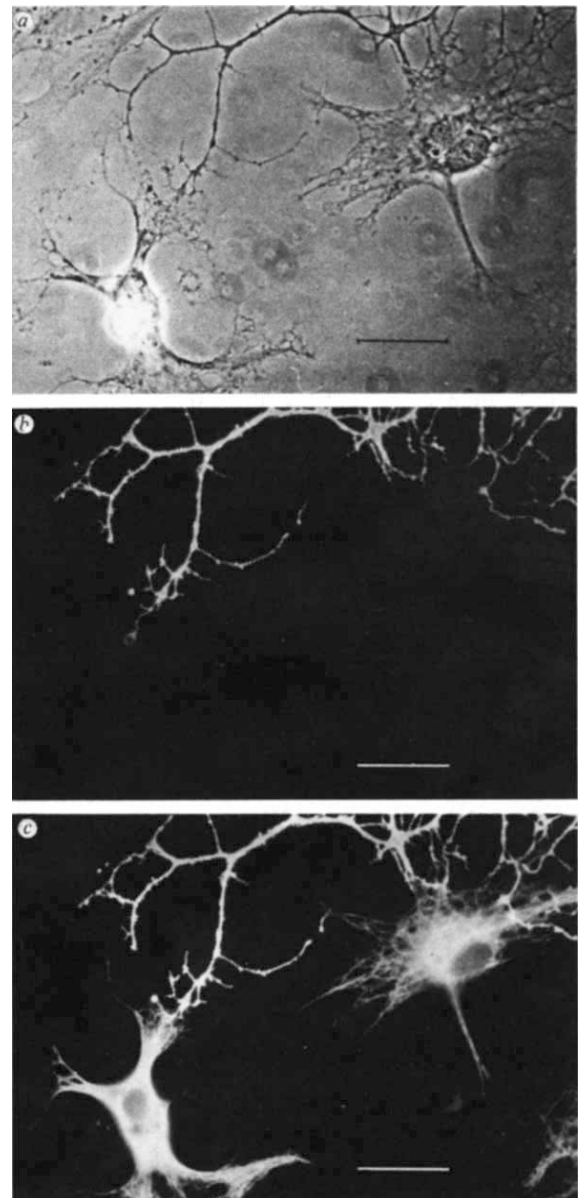
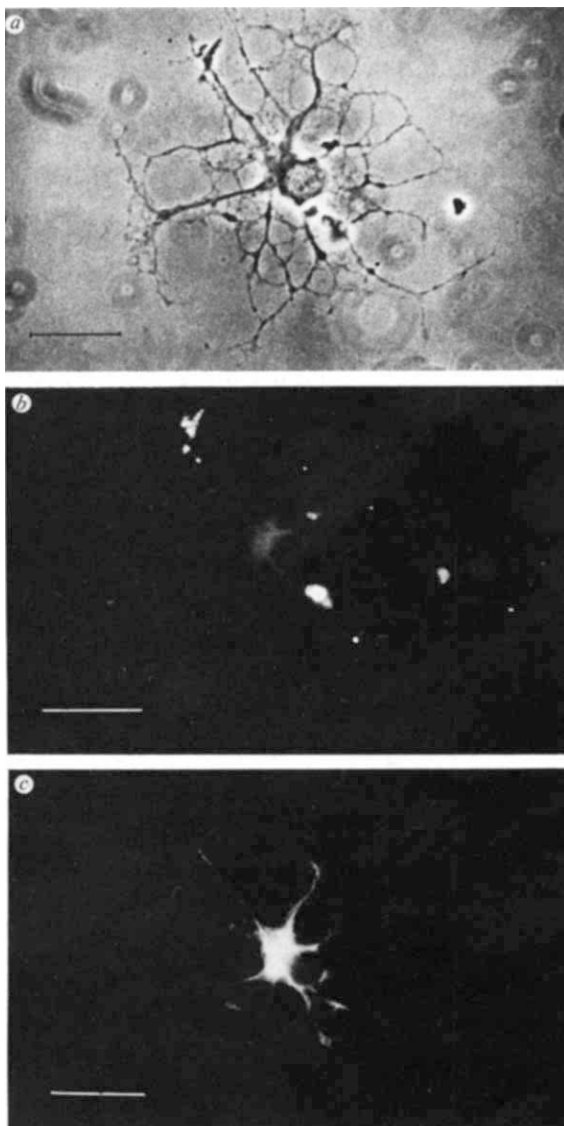
To determine if the cells labelled with R anti-GC serum were also GFAP<sup>+</sup>, living cells were first exposed to R anti-GC serum, followed by G anti-RIg-Rd and then fixed in acid-alcohol. After washing, the fixed cells were incubated in R anti-GFAP serum followed by G anti-RIg-Fl. Although the G anti-RIg-Fl detected both the R anti-GFAP and R anti-GC antibodies (Fig. 4), the distinctive intracellular fibrillar staining of the glial filaments with anti-GFAP serum usually made it easy to distinguish from the characteristic surface labelling of the anti-GC serum. In such



double labelling experiments, none of the GFAP<sup>+</sup> cells were labelled by the anti-GC serum and the GC<sup>+</sup> cells were GFAP<sup>-</sup> (Figs 3 and 4). In preliminary experiments, this was confirmed using mouse anti-GFAP and rabbit anti-GC sera, where the labelling was unambiguous (R. Pruss, unpublished observations).

These results strongly suggested that the GC<sup>+</sup> cells in optic nerve cultures were oligodendrocytes. Since virtually all of the process-bearing cells in these cultures labelled with either anti-GC or anti-GFAP (but never both), it is likely that all, or the great majority of, oligodendrocytes in these cultures were GC<sup>+</sup>. To determine if any other cell type was GC<sup>+</sup>, we looked, by indirect immunofluorescence, at dissociated primary cell cultures of neonatal rat sciatic nerve, muscle and leptomeninges and failed to find any GC<sup>+</sup> cells. Moreover, in similar cultures of cerebellum, corpus callosum and cerebral cortex, a small proportion of the cells were GC<sup>+</sup> and all of these had the typical oligodendrocyte-like morphology seen in optic nerve cultures and were GFAP<sup>-</sup> in double labelling experiments; cells in these

**Fig. 3** GFAP<sup>+</sup>/GC<sup>-</sup> cell (putative fibrous astrocyte) in optic nerve culture from 8-d-old rats. After 5 d in culture, the cells were labelled with R anti-GC (1:20) followed by G anti-RIg-Rd (1:50) and fixed in acid-alcohol. After washing, they were labelled with R anti-GFAP (1:50) followed by G anti-RIg-Fl (1:100) and viewed with phase contrast (top), rhodamine (middle) and fluorescein (bottom) optics. Scale bar, 10  $\mu$ m.



**Fig. 4** Two GFAP<sup>+</sup>/GC<sup>-</sup> cells (putative astrocytes) and GC<sup>+</sup> process of a putative oligodendrocyte (whose cell body is not in field) in the same culture as that of Fig. 3, viewed with phase contrast (top), rhodamine (middle) or fluorescein (bottom) optics. Bar represents 10  $\mu$ m.

cultures with typical neuronal morphology and which bound tetanus toxin (previously shown to bind primarily to neurones<sup>16,17</sup>) were all GC<sup>-</sup>.

It was surprising to find that cultured Schwann cells, which are known to make myelin in peripheral nerves, were GC<sup>-</sup>. To check that our antisera could detect GC in both central and peripheral myelin, we examined unfixed frozen sections of adult rat optic and sciatic nerves by indirect immunofluorescence. In both cases myelin was intensely labelled by the R anti-GC sera, whereas various normal rabbit sera and anti-GC serum adsorbed with myelin or galactocerebroside were inactive. There are several possible explanations for this paradox: (1) Schwann cells may not synthesise large amounts of GC before they myelinate; (2) GC may be present in the plasma membranes of cultured Schwann cells but inaccessible to antibody; or (3) Schwann cells may normally synthesise GC *in vivo* but stop doing so in culture. Preliminary observations suggest that the third explanation is correct.

We conclude that galactocerebroside (GC) can serve as a specific cell-surface antigenic marker for rat oligodendrocytes in culture. Preliminary results suggest that it can also serve this function for mouse and human oligodendrocytes. Previous studies have demonstrated that GC is a major component of cell suspensions prepared from adult bovine brain which are enriched for oligodendrocytes (ref. 18 and R.P.L. *et al.*, unpublished) and of two out of six clones derived from a mouse glial tumour<sup>19</sup>. Thus anti-GC serum should prove valuable for identifying oligodendrocytes and their tumours, for separating oligodendrocytes from other cell types and for studying their development and properties. In addition, our studies, and those of others<sup>20-22</sup>, suggest that GFAP is a specific intracellular marker for both protoplasmic and fibrous astrocytes *in vitro*. Previously, tetanus toxin was shown to bind principally to neurones in neural cell cultures<sup>16,17</sup>. Together, these three markers can be used to identify the three major cell types of the central nervous system in a number of different species.

We thank Drs D. Dahl and A. Bignami for the gift of anti-GFAP serum, Janet Winter and Jeremy Brookes for providing the purified myelin, and Ann Hornby-Smith for technical assistance.

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## Localisation and characterisation of aminopeptidases in the CNS and the hydrolysis of enkephalin

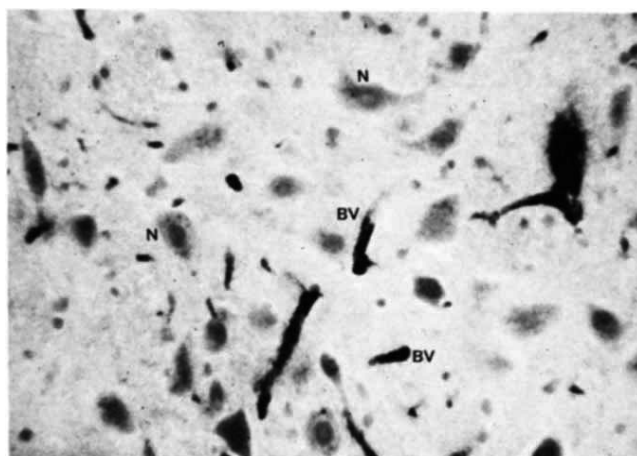
THE possible neurotransmitter or neuromodulatory roles of several peptides found in the mammalian central nervous system (CNS) has prompted interest in peptidase enzymes which might be involved in their formation and degradation. Particular interest has focussed on the arylamidases which have been implicated in the formation and destruction of peptides such as angiotensin II, kinins and the enkephalins<sup>1-3</sup>. We report here selective localisation of some arylamidases to particular cells and regions of the mouse CNS. The difference in substrate specificities suggests that particular enzymes could play a select role in the metabolism of particular peptides, whereas others are probably involved more generally with protein metabolism.

We have used a histochemical method and polyacrylamide gel electrophoresis (PAGE)<sup>4</sup> both based on the fact that  $\beta$ -naphthylamine enzymatically liberated from amino acid substituted naphthylamides can be coupled with an azo salt, in this case Fast Black K, to form an insoluble blue dye which indicates sites of enzyme activity.

Histochemically, two principal types of staining were seen depending on whether alanine  $\beta$ -naphthylamide or leucine  $\beta$ -naphthylamide was used as substrate. With the former, staining was confined to blood vessels (Fig. 1), but with the latter there was also heavy staining of neuronal cell bodies (Fig. 2) in many of the cranial nerve nuclei in the pons and medulla, as well as regional staining of particular areas such as the paraventricular and supraoptic nuclei. It is not possible to ascertain the precise site of  $\beta$ -naphthylamine production but it may be associated with the neurophil in these regions. Other areas staining in this way included the medial habenular nucleus, the ventral and medial thalamic nuclei and medial hypothalamic nuclei, as well as the CA3 pyramidal cell layer of the hippocampus. The significance of the enzyme activity in these areas is not known. A similar pattern of staining is seen in the rat (personal observation).

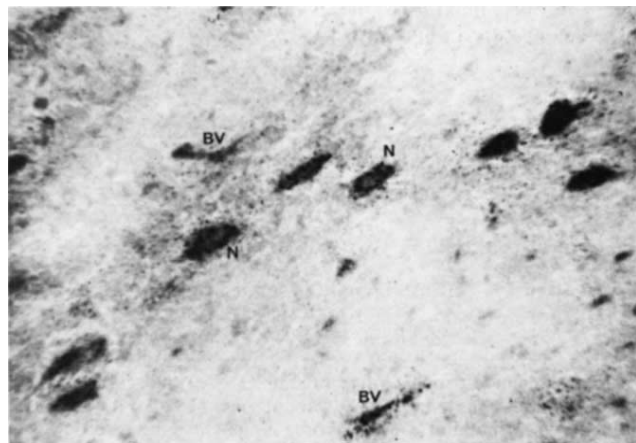
As the histochemical method does not distinguish between particular enzymes, our preliminary histochemical study was combined with an analysis of the number of enzymes present in mouse brain using PAGE. We have demonstrated the presence of at least seven arylamidases (Fig. 3) numbered 1-7 in decreasing order of electrophoretic mobility. Some of these, in

**Fig. 1** Nucleus retrofacialis stained for arylamidase activity using alanine  $\beta$ -naphthylamide as substrate and Fast Black K as the coupling salt<sup>4</sup>. In this monochrome photograph the reaction product which in reality is deep blue, appears black and is confined solely to blood vessels (BV). Neuronal cell bodies (N), which have been counterstained with acetocarmine, appear paler and are devoid of reaction product.





the conditions of the staining reaction, showed considerable overlap in amino acid specificity, whereas others were more specific for the amino acids they would cleave, either from the substituted naphthylamides or from the N-terminal end of a peptide chain. Enzyme 3, which seemed from the gels to have the predominant activity, had a broad specificity and would cleave the peptide bond of naphthylamide substrates which are substituted with most of the neutral amino acids, the basic amino acids lysine and arginine, or tyrosine and histidine. It did not, however, hydrolyse to any detectable extent the peptide bond of naphthylamide substrates in which the amino acid substituent is glycine, aspartate, glutamate or proline. The pH optimum was 6.5–7.0 for the partially purified enzyme, which was completely inactivated by freezing and thawing, susceptible to thiol reagents and markedly inhibited by chlorhexidine



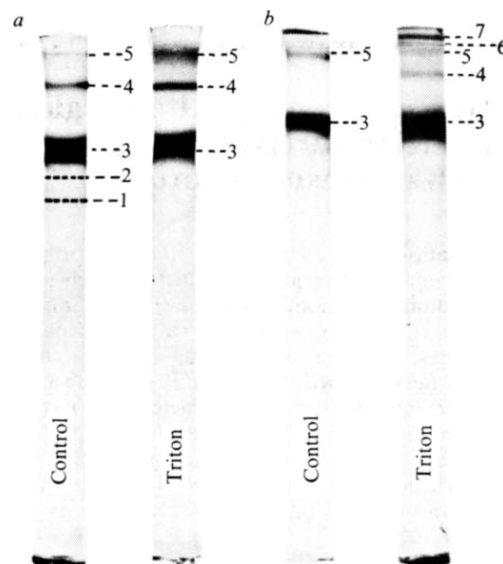
**Fig. 2** Neurones of the nucleus vestibularis superior stained for arylamidase activity using leucine  $\beta$ -naphthylamide as substrate and Fast Black K as the coupling salt<sup>4</sup>. The dark reaction product is localised not only to blood vessels (BV) but also to the neuronal cell bodies (N), which gives them a dark granular appearance. This is in contrast to the complete lack of such neuronal staining when the alanine derivative is used as substrate (Fig. 1).

acetate and 1 mM  $Zn^{2+}$ ,  $Mn^{2+}$ , EDTA and puromycin. Enzyme 5 was similar with respect to the effects of these inhibitors and it also had a broad specificity although its pH optimum was 7.5.

Other enzymes seemed to be more specific, enzyme 1 for basic amino acids, and enzyme 2 for dipeptide sequences, in this case arginyl-arginine. Other dipeptide sequences have not been tested with this enzyme. Enzyme 4, which was detectable after freezing and thawing the brain or after Triton X-100 treatment of whole brain homogenate, showed a preference for neutral amino acid substituted naphthylamide substrates, in particular leucine rather than alanine. Enzymes 6 and 7, which were detected after treatment of brain homogenate with 1% Triton X-100, also seemed to show a preference for neutral amino acid substituted naphthylamides in particular alanine rather than leucine.

Because the staining conditions for the visualisation of enzyme activity on gels were identical to those used histochemically, some analogies can be seen between the staining of particular enzymes on the gels and the staining of specific elements in brain. The two sorts of staining with the two substrates suggest the presence in mouse brain of an enzyme, associated with cell bodies of cranial nerve nuclei and other regions, which in the conditions of the staining reaction preferentially hydrolysed the leucine substrate. Figure 3 shows that enzyme 4 had this property, suggesting that it may be localised in regions of the CNS. For enzymes 3 and 5 an overall distribution was suggested by their broad specificities and the consistent staining of blood vessels and choroid plexus which was found using a variety of naphthylamide substrates.

The high blood vessel activity indicates that a number of earlier studies<sup>1–3</sup> on aminopeptidase distribution in brain



**Fig. 3** Bands of arylamidase activity detectable on 9.5% polyacrylamide gels before and after Triton X-100 treatment of whole brain homogenate. Following Triton treatment, the brain homogenate was centrifuged at 100,000g to obtain a supernatant fraction which was subsequently applied to the gels. As control, Triton was added to a supernatant fraction from brain which had first been frozen and thawed as a check that Triton itself did not alter the mobility of the enzymes. Subsequent staining of the gels, using leucine  $\beta$ -naphthylamide as substrate (a) and alanine  $\beta$ -naphthylamide as substrate (b), each at a concentration of 0.1 mg ml<sup>-1</sup> with Fast Black K as the trapping salt results in the following staining patterns. Using the alanine derivative, three additional bands of activity are detectable in the soluble supernatant fraction from Triton-treated whole brain homogenate enzymes 4, 6 and 7. Enzyme 4, which only slowly hydrolyses the alanine substrate, is now in sufficient concentration on the gel to show as a faint band. Using the leucine derivative as substrate no additional bands of activity are detectable following Triton treatment. Enzyme 4, however, seems to be solubilised quite effectively and now stains intensely on gel (a). Enzymes 1 and 2, which can be demonstrated using arginine  $\beta$ -naphthylamide and arginyl-arginine  $\beta$ -naphthylamide respectively as substrates, are not shown on the above gels. The relative position of these two enzymes is indicated, however, by the dotted lines in a.

regions and subfractions may require reconsideration. Because of inherent difficulties in biochemical subfractionation techniques, enzyme activity detectable in 'synaptosomal' or 'microsomal' fractions may largely be due to contaminating fragments from vascular elements and may have no role in the regulation of activity of particular neuropeptides.

Enzyme 3, for example, hydrolysed enkephalin by removing the N-terminal tyrosine. Hydrolysis of the Gly-Gly bond was rate limiting and the reaction was inhibited by leucine  $\beta$ -naphthylamide. Thus, it is likely that enzyme activity associated with blood vessels contributes most of the enkephalin-hydrolysing activity found in brain homogenates. This emphasises the importance of careful localisation studies before assigning a regulatory role to any aminopeptidase activity associated with particular subfractions or regions of brain.

S.G.S. is an MRC Training Fellow.

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Received 25 April; accepted 3 July 1978.

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## New factor released by cultured glioma cells supporting survival and growth of sensory neurones

PROCESS formation can be induced in clonal neuroblastoma cells by a macromolecule termed glial factor which has been identified in medium conditioned by cultured C-6 glioma cells<sup>1</sup>. Fibre outgrowth can also be elicited in cultures of peripheral sensory neurones<sup>2</sup> by the well-characterised mouse submaxillary gland nerve growth factor (NGF)<sup>3</sup>, which has been shown to be a different entity from glial factor<sup>4</sup>. We report here that glioma-conditioned medium (GCM), the source of glial factor, can support both survival and fibre formation of isolated chick sensory neurones and that neither NGF nor glial factor are responsible for this effect.

We conclude that C-6 glioma cells release into the culture medium a substance, probably a polypeptide, which can support the survival and process formation of dissociated neurones from chick embryo sensory ganglia. This substance is neither NGF nor the previously described glial factor<sup>1</sup>. Hitherto NGF has been the only factor known to have the ability to support the survival of peripheral sensory neurones during a critical phase of their development. The existence of a factor other than NGF with this property means that in this respect at least NGF can no longer be considered unique.

**Table 1** Comparison between the effects of NGF and GCM on the survival of chick sensory neurones

| a | Control | NGF (1 ng/ml <sup>-1</sup> ) | NGF (1 ng/ml <sup>-1</sup> )<br>+ Ab (100 ng/ml <sup>-1</sup> ) | GCM                                | GCM + Ab (100 ng/ml <sup>-1</sup> ) |                                                  |
|---|---------|------------------------------|-----------------------------------------------------------------|------------------------------------|-------------------------------------|--------------------------------------------------|
|   |         | 300 ± 150                    | 2,500 ± 700                                                     | 350 ± 150                          | 1,300 ± 300                         | 1,350 ± 250                                      |
| b | Control | NGF (1 ng/ml <sup>-1</sup> ) | GCM                                                             | GCM + Ab (10 µg/ml <sup>-1</sup> ) | GCM + Pronase                       | Inact. Pronase<br>+ NGF (1 ng/ml <sup>-1</sup> ) |
|   |         | 950 ± 200                    | 5,250 ± 350                                                     | 3,950 ± 500                        | 4,500 ± 650                         | 1,950 ± 300<br>4,250 ± 850                       |

The results in *a* and *b* are from two separate experiments. Cell numbers shown are the mean of triplicate determinations ± s.d. and represent the total number of surviving neurones per 35-mm dish. Nine per cent of the surface of the dish was counted. Cultures were prepared by adding 1.0 ml of cell suspension (25,000–35,000 cells in F-14 medium) to 1.0 ml of Dulbecco's modified Eagle's medium (DMEM) containing (where indicated) twice the final amount of NGF and/or NGF antibodies (Ab). Both F-14 and DMEM contained 5% (v/v) fetal calf serum and 2% (v/v) chicken serum (Gibco). On testing GCM, it was substituted for an equivalent volume of DMEM. GCM was obtained by culturing C-6 glioma cells as described before<sup>6</sup>, except that after serum-free incubation, cells were returned to DMEM containing 3% fetal calf serum. After 72 h in this medium, cells were again cultured in serum-free medium for three consecutive 24-h periods. The conditioned medium obtained during the last period was the most active and was used in the experiments. At that time, the glioma cells had reached a density of  $9 \times 10^8$  per roller flask and the protein concentration of the conditioned medium was 10–20 µg/ml<sup>-1</sup>. The GCM was centrifuged and used without filtration. NGF was purified essentially according to Bocchini and Angeletti<sup>7</sup>. Maximal fibre outgrowth was produced in the chick dorsal root bioassay at a concentration of 5–10 ng/ml<sup>-1</sup> (ref. 8). Antibodies were purified by passing the NGF-antisera over an NGF-Sepharose column as previously described<sup>9</sup>. In *b*, GCM was exposed to Pronase (Serva) (10 µg/ml<sup>-1</sup>, 30 min, 37 °C) and subsequently heated for 30 min at 60 °C. Such heat treatment did not reduce the activity of GCM but did inactivate the Pronase, as shown. DMEM was similarly treated (Pronase followed by heat inactivation) before the addition of NGF. The number of surviving neurones is not different from that for DMEM + NGF untreated control.

Dorsal root ganglia from 10–12-d-old chick embryos were dissociated and plated on collagen-coated dishes, after a pre-plating period to eliminate most non-neuronal cells as before<sup>5</sup>. Surviving neurones (defined as process-bearing cells with highly refractile soma) were counted after 48 h. In the absence of either NGF or GCM, less than 5% of the cells plated survived. The addition of either GCM or NGF led to a marked increase in the number of surviving neurones. As Table 1 shows, the effect of NGF could be abolished by specific antibodies to NGF. In contrast, the increased survival of neurones promoted by GCM was not blocked by the NGF antibodies, even when the concentration of antibody used was 100 times greater than that required to block the effect of NGF (Table 1*b*).

Partially purified glial factor (eluted as a single peak from a DEAE-cellulose column, R.M.L. and D.M., unpublished results) did not support the growth of the isolated sensory neurones in spite of an increase in specific activity of more than 80-fold as determined by neuroblastoma bioassay. Therefore we conclude that the support of survival and the induction of fibre outgrowth in sensory neurones were due to a factor other than that responsible for neuroblastoma cell process formation, that is, something other than glial factor.

Digestion of GCM with Pronase markedly reduced the ability of GCM to support the survival of sensory neurones (Table 1*b*). Similar results were obtained after digestion with α-chymotrypsin, suggesting that this factor might be a polypeptide. Further characterisation is in progress.

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Received 24 April; accepted 20 June 1978.

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## Synthetic $\alpha$ -adrenergic agonists are potent $\alpha$ -adrenergic blockers in human platelets

ADRENALINE- and noradrenaline-induced aggregation of human platelets<sup>1-3</sup> is blocked by the  $\alpha$ -adrenergic blocking agents, dihydroergotamine, phentolamine, phenoxybenzamine and dibenamine<sup>4-6</sup>, suggesting the involvement of  $\alpha$ -adrenergic receptors. We have demonstrated that adrenaline and noradrenaline inhibit adenylate cyclase (EC 4.6.1.1) in lysates<sup>7</sup> and particles<sup>8</sup> of human platelets, an effect also blocked by such  $\alpha$ -adrenergic antagonists. Synthetic phenylethylamine and imidazoline derivatives that act as  $\alpha$ -adrenergic agonists in various other tissues neither induced primary platelet aggregation nor inhibited adenylate cyclase when added at concentrations up to 100  $\mu$ M<sup>9</sup>.  $\alpha$ -Adrenergic receptors have been identified in human platelets by binding studies using tritiated dihydroergocryptine<sup>10,11</sup> or dihydroergonine<sup>12,13</sup>. The sites to which the dihydrogenated ergot alkaloids were bound showed the typical pattern of an  $\alpha$ -adrenergic receptor with respect to displacement by adrenergic agonists and antagonists. However, there have been some discrepancies between the binding affinities of some  $\alpha$ -adrenergic agonists and their biological efficacies concerning platelet aggregation and inhibition of adenylate cyclase. The  $\alpha$ -adrenergic agonist, clonidine, displaced dihydroergocryptine from the binding sites with similar<sup>10</sup> or even a 10-fold higher affinity<sup>11</sup> relative to adrenaline, but clonidine (up to 100  $\mu$ M) was ineffective in inducing primary platelet aggregation and inhibition of adenylate cyclase<sup>9</sup>. Similar dissociation constants were obtained for phenylephrine and noradrenaline in binding studies<sup>12</sup>, but in contrast to the natural compound, phenylephrine (up to 100  $\mu$ M) was unable to induce a biological response<sup>9</sup>. We observed similar discrepancies with methoxamine, xylometazoline and oxymetazoline<sup>12,13</sup>. Agents that undergo the primary reaction of high affinity-binding to the receptor but are unable to induce secondary reactions in the form of biological responses should act as antagonists. We have tested this hypothesis and show here that various agents that are potent  $\alpha$ -adrenergic agonists in various other systems are  $\alpha$ -adrenergic antagonists in human platelets with respect to adrenaline-induced aggregation and inhibition of adenylate cyclase.

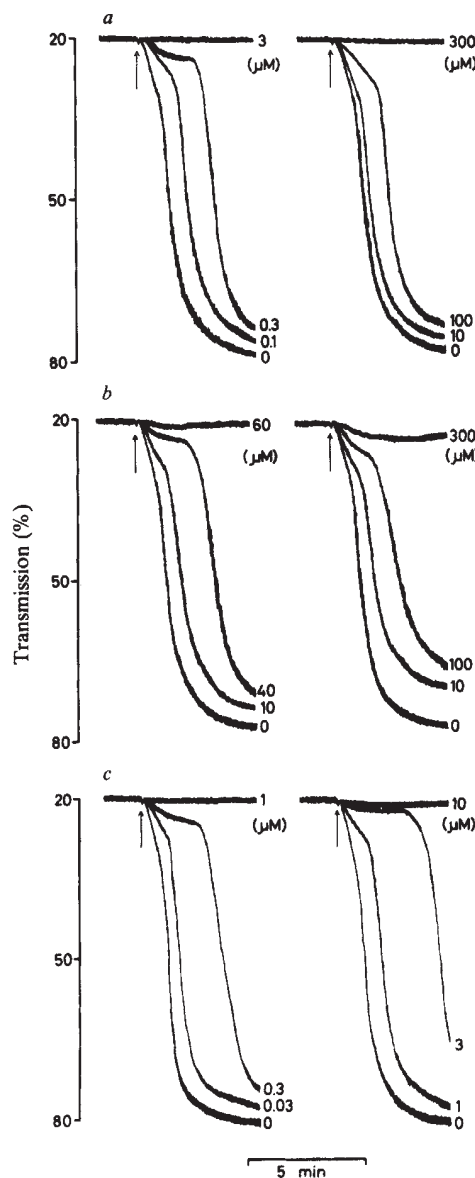
Figure 1 shows the effects of phenylephrine, methoxamine, clonidine and xylometazoline on adrenaline-induced platelet aggregation in comparison with the effects of two typical  $\alpha$ -adrenergic antagonists, phentolamine and phenoxybenzamine. Phentolamine completely blocked adrenaline-induced aggregation at 3  $\mu$ M, and its effect was half-maximal at about 0.3  $\mu$ M. Phenoxybenzamine, on a molar basis, was much less potent than phentolamine. Similarly, xylometazoline, clonidine, phenylephrine and methoxamine dose-dependently inhibited adrenaline-induced aggregation, with complete blockade at 1, 10, 60 and 300  $\mu$ M, respectively. The potency of xylometazoline was similar, on a molar basis, to that of phentolamine; clonidine was somewhat less potent, and compared with xylometazoline, about 50- and 300-fold higher concentrations of phenylephrine and methoxamine, respectively, were required to inhibit adrenaline-induced aggregation. Phenylephrine was still more effective than the typical  $\alpha$ -adrenergic antagonist, phenoxybenzamine, and methoxamine had about the same potency as phenoxybenzamine. By themselves, xylometazoline, clonidine, phenylephrine and methoxamine (up to 1 mM) neither induced primary platelet aggregation nor increased the aggregation induced by adrenaline added at threshold concentrations in the absence and presence of the  $\beta$ -adrenergic antagonist, pindolol (10  $\mu$ M) (not shown).

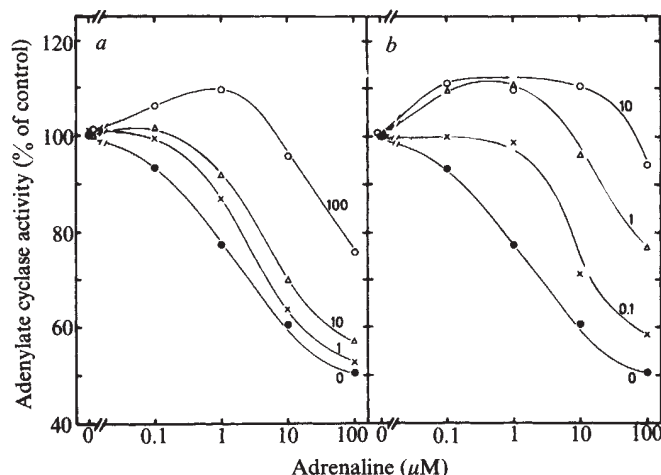
The order of potency of these agents in inhibiting adrenaline-induced aggregation agreed well with their effects on dihydroergocryptine or dihydroergonine binding. The dis-

sociation constants found in the binding studies were 0.01  $\mu$ M (ref. 13), 0.02  $\mu$ M (ref. 11), 0.9  $\mu$ M (ref. 11) and 5  $\mu$ M (ref. 13) for xylometazoline, clonidine, phenylephrine and methoxamine, respectively.

In parallel experiments, we studied the effects of phenylephrine and xylometazoline on adrenaline-induced inhibition of platelet adenylate cyclase. Adrenaline reduced adenylate cyclase activity maximally by about 50%, with a half-maximal effect at about 1  $\mu$ M<sup>7,8</sup> (Fig. 2). Phenylephrine and xylometazoline, up to 100  $\mu$ M, had no significant effect on enzyme activity in the absence of adrenaline. However, in its presence, these agents inhibited the adrenaline effect and shifted the dose-response curve for adrenaline to the right. Xylometazoline, which was much more effective than phenylephrine in inhibiting adrenaline-induced aggregation, was also about 100-fold more potent in reversing adrenaline-induced

**Fig. 1** Effects of *a*, phentolamine (left side), phenoxybenzamine (right side); *b*, phenylephrine (left), methoxamine (right); *c*, xylometazoline (left) and clonidine (right) on adrenaline-induced aggregation in citrated human platelet-rich plasma. Aggregation was monitored at 37 °C in 250- $\mu$ l plastic cuvettes with a magnetic stirrer (1,000 r.p.m.) in an ELVI aggregometer according to Born<sup>15</sup>. The agents were added at the indicated concentrations 1 min before the addition of 3  $\mu$ M adrenaline, which is indicated by the arrows.





**Fig. 2** Effects of phenylephrine and xylometazoline on adrenaline-induced inhibition of adenylate cyclase in platelet particles. Adenylate cyclase activity was assayed as described<sup>8</sup>, with 0.1 mM [ $\alpha$ -<sup>32</sup>P]ATP (0.8  $\mu$ Ci per tube), 5 mM MgCl<sub>2</sub>, 1 mM 3-isobutyl-1-methylxanthine, 0.1 mM ethyleneglycol-bis( $\beta$ -aminoethylether)-N,N'-tetraacetic acid, 5 mM creatine phosphate and 0.4 mg per ml of creatine kinase in 50 triethanolamine-HCl, pH 7.4, for 20 min at 37 °C with about 50  $\mu$ g of platelet protein. GTP (30  $\mu$ M) and pindolol (10  $\mu$ M) were present in each condition. The ordinate indicates the adenylate cyclase activity as % of control activity at the indicated concentrations of adrenaline with or without the concurrent presence of phenylephrine (a) or xylometazoline (b) at the indicated concentrations ( $\mu$ M). Control activity, observed in the absence of any agent, was 26.9 pmol cyclic AMP per mg protein per min.

inhibition of adenylate cyclase. This means that phenylephrine and xylometazoline, similar to their effects on aggregation, acted as  $\alpha$ -adrenergic antagonists in the adenylate cyclase system. In the presence of high concentrations of xylometazoline and phenylephrine, the inhibition of adenylate by the  $\alpha$ -adrenergic component of adrenaline was not only completely reversed, but there was also a small stimulatory effect of adrenaline on adenylate cyclase, probably through its  $\beta$ -adrenergic component<sup>9</sup>.

Of the many  $\alpha$ -adrenergic agonists studied we found that only adrenaline and noradrenaline were able to induce primary platelet aggregation and inhibition of adenylate cyclase<sup>9</sup>, whereas these natural agonists and all the synthetic  $\alpha$ -adrenergic agonists studied were able to displace dihydroergocryptine or dihydroergonine binding with high affinity<sup>10,11,13</sup>. These findings and the data presented above indicate that, in the platelet system, only the naturally occurring compounds, adrenaline and noradrenaline, are  $\alpha$ -adrenergic agonists, whereas the synthetic  $\alpha$ -adrenergic agonists are antagonists. With such a narrow profile of active stimuli, the platelet  $\alpha$ -adrenergic receptor differs completely from  $\alpha$ -adrenergic receptors found in other tissues. In addition to the  $\alpha_1$ -(post-synaptic) and  $\alpha_2$ -(presynaptic) subclasses of the  $\alpha$ -adrenergic receptor<sup>14</sup>, a third type of  $\alpha$ -adrenergic receptor apparently exists, through which so far only the naturally occurring catecholamines are capable of triggering cellular responses.

This work was supported by the Deutsche Forschungsgemeinschaft. I thank Miss Gabriele Gabel for technical assistance.

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Received 20 April; accepted 19 June 1978.

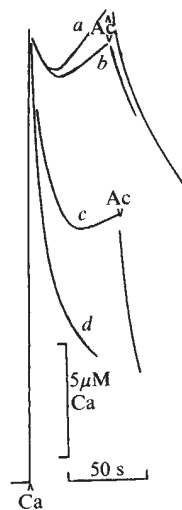
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## The importance of CO<sub>2</sub> for Ca<sup>2+</sup> uptake by some mitochondria

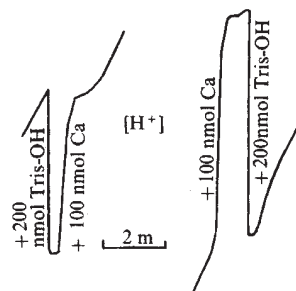
METABOLISING mammalian mitochondria normally will take up a considerable (30-80 nmol per mg protein) quantity of Ca<sup>2+</sup> when no anion, such as phosphate or acetate, has been added to move in and balance the charges on the Ca<sup>2+</sup> accumulated<sup>1,2</sup>. This has been attributed to reaction of the Ca<sup>2+</sup> with membrane components. Recently<sup>3,4</sup> the importance of adventitious phosphate, always present in mitochondrial suspensions<sup>3</sup>, has been recognised, and it has been shown that inhibition of phosphate movement lessens<sup>3,5</sup>, or can abolish, Ca<sup>2+</sup> uptake<sup>4</sup>. Carbon dioxide is a further source of anion to accumulate with Ca<sup>2+</sup> in mitochondria possessing carbonic anhydrase activity<sup>6</sup>. The importance of this process, which readily proceeds using the dissolved CO<sub>2</sub> already present in the system, is now illustrated using a sensitive indicator of Ca<sup>2+</sup> movement. Also, the production of 2H<sup>+</sup> per Ca<sup>2+</sup> accumulated as CaCO<sub>3</sub> can account for the increase in the ratio H<sup>+</sup>:Ca<sup>2+</sup>, which is observed when phosphate uptake is inhibited.

When Ca<sup>2+</sup> is taken up, about 1H<sup>+</sup> usually appears in the medium per Ca<sup>2+</sup> taken up<sup>1,2</sup> although a higher ratio is some-



**Fig. 1** Effect of CO<sub>2</sub> on Ca<sup>2+</sup> by rat liver mitochondria. The concentration of free Ca<sup>2+</sup> is indicated by the absorbance difference (685-675 nm) of the metallochrome indicator Arsenazo III. The mitochondrial suspension (1.4 mg protein ml<sup>-1</sup>) was supplemented with mersalyl (20  $\mu$ M) to inhibit phosphate movement, and with oligomycin (2  $\mu$ g ml<sup>-1</sup>) to prevent hydrolysis of internal ATP providing phosphate. The addition of CaCl<sub>2</sub> to 20  $\mu$ M caused an increase of absorbance which diminished if Ca<sup>2+</sup> moved into the suspended particles. Transfer of Ca<sup>2+</sup> was almost absent when either Diamox (20  $\mu$ M) was also present, to prevent hydration of adventitious CO<sub>2</sub> (curve a), or when the medium was pre-gassed with pure O<sub>2</sub> to remove CO<sub>2</sub> (curve b). Without pre-gassing there was an appreciable movement of Ca<sup>2+</sup>, whereas pre-gassing with 5% CO<sub>2</sub>:95% O<sub>2</sub> resulted in considerably more uptake (curve d). Addition of acetate (to 10 mM) made at Ac in the first three experiments showed that Ca<sup>2+</sup> uptake could subsequently proceed when a suitable anion was provided. The procedures for measurement and preparation of the mitochondria are as described in ref. 4.





**Fig. 2** Proton liberation in the medium associated with uptake of  $\text{Ca}^{2+}$  from carbonated medium. The trace obtained from a pH electrode shows the increasing acidity of a suspension of rat liver mitochondria ( $1.1 \text{ mg protein ml}^{-1}$ ) which was gassed with 5%  $\text{CO}_2$ :95%  $\text{O}_2$ ; addition of 100 nmol  $\text{CaCl}_2$ , with pH adjusted to that of the suspension at that time (7.2), shifted the pH immediately; this was then cancelled out by the addition of 200 nmol base. The same results were obtained when these additions were made in either order. A  $\text{Ca}^{2+}$ -sensitive electrode was used to confirm that all the added  $\text{Ca}^{2+}$  was removed from the solution.

times obtained<sup>7</sup>. It was suggested<sup>8</sup> that the acid arises from the change of ionisation of the phosphate derived from the medium as it accompanies the  $\text{Ca}^{2+}$  in a proportion close to that expected for  $\text{Ca}_3(\text{PO}_4)_2$  (ref. 9). The contributions, both to provision of anion to accumulate with the  $\text{Ca}^{2+}$  and to  $\text{H}^+$  production, from the  $\text{CO}_2$  hydration process occurring in liver mitochondria, are only fully exposed when movement of phosphate has been inhibited with an agent such as mersalyl or *N*-ethylmaleimide. Additionally, generation of internal phosphate by hydrolysis of endogenous ATP can account for some  $\text{Ca}^{2+}$  uptake and  $\text{H}^+$  production unless oligomycin has been used to deplete the ATP and to inhibit ATPase<sup>4</sup>. With these precautions, liver mitochondria will take up very little  $\text{Ca}^{2+}$ , as is shown in Fig. 1, where trace (a) shows the small and transient uptake of  $\text{Ca}^{2+}$  obtained in the presence of Diamox (to inhibit carbonic anhydrase activity) and trace (b) the similar result obtained if the medium has been pre-gassed with pure  $\text{O}_2$  to remove dissolved  $\text{CO}_2$ . Trace (c) shows the result obtained with untreated solution, with which about one-third of the applied  $\text{Ca}^{2+}$  moves into the mitochondria to give an uptake of 6 nmol per mg protein. If the medium was briefly gassed with a 5%  $\text{CO}_2$ :95%  $\text{O}_2$  mixture the  $\text{Ca}^{2+}$  uptake was increased considerably (curve d). As a check that the mitochondria could take up the  $\text{Ca}^{2+}$  when given a penetrant anion, a subsequent addition of acetate was made in the experiments (Ac).

In other experiments in which  $\text{Ca}^{2+}$  additions were made to a suspension in a medium containing mersalyl and oligomycin, and which had been exposed to 5%  $\text{CO}_2$ :95%  $\text{O}_2$ , it was confirmed that the predictable yield of  $2\text{H}^+$  appeared in the medium per  $\text{Ca}^{2+}$  taken up (Fig. 2). Since this is about double that calculated for movement of  $\text{Ca}^{2+}$  with phosphate, the variable values of  $\text{H}^+:\text{Ca}^{2+}$  obtained with media containing uncontrolled concentrations of endogenous phosphate and dissolved  $\text{CO}_2$  can be understood. In this context, the observations that inhibitors of phosphate movement increased both the  $\text{H}^+:\text{O}$  per site ratio obtained when small pulses of oxygen were added to a previously anaerobic suspension supplemented with  $\text{CaCl}_2$  (ref. 10) as well as the ratio  $\text{H}^+:\text{Mn}^{2+}$  taken up by mitochondria<sup>11</sup> are probably explicable.

I thank the British Heart Foundation for a grant and Dr M. C. W. Evans of the Botany Department, University College London, for use of a spectrophotometer.

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Received 24 May; accepted 10 July 1978.

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## Resolution of X and Y spermatids by pulse cytophotometry

SEPARATION of sperm bearing X and Y chromosomes is limited by, among other difficulties, the inability to distinguish between the two cell types. The two types of sperm can be distinguished only in man because the Y chromosome is highly fluorescent when stained with quinacrine mustard<sup>1</sup>. Because of the greater length of the X chromosome than the Y chromosome, it is expected that X-bearing sperm will have a greater DNA content. Based on chromosome length measurements<sup>2</sup>, the difference in DNA content between X-bearing sperm of the mouse (*Mus musculus*), which carry 19 autosomes plus the X chromosome, and the Y-bearing sperm, which carry the autosomes plus the Y chromosome, is expected to be 3.4%. Distinguishing X and Y sperm on the basis of this difference requires a method of measurement of DNA content with high precision. We have been able to resolve the two classes of spermatids by pulse cytophotometry (that is, flow microfluorometry).

Previous attempts to detect this difference have been unsuccessful for several reasons<sup>3,4</sup>. First, measurement of DNA content with a coefficient of variation (CV) of about 1% is required to distinguish cells with such a small difference in DNA content. It is only through recent improvements in instrumentation<sup>5</sup>, cell preparation methods and staining procedures<sup>6,7</sup> that this resolution could be achieved with pulse cytophotometry. Second, accurate measurement of the DNA of mature sperm is limited by the following factors: the condensed nature of the sperm chromatin, and the presence of highly basic proteins, do not permit stoichiometric staining of DNA with most conventional procedures<sup>3</sup>. Furthermore, the asymmetric shape and high refractive index of spermatozoa result in broadened and asymmetric DNA distributions, especially when analysed in flow machines with small angle fluorescence excitation and measurement. We have therefore chosen to analyse first the DNA distributions of testicular spermatids. The early spermatids possess round nuclei and histone-containing chromatin, while late spermatids have elongated nuclei and unique spermatid basic proteins<sup>8,9</sup>.

Testicular cell suspensions were prepared from sexually mature mice by mincing the tubules and subsequent treatment with crude trypsin and DNase<sup>10,11</sup>. Some of the cell suspensions were separated by centrifugal elutriation<sup>12</sup>. Fractions enriched in round spermatids (fraction 2)<sup>13</sup> and in pachytene spermatocytes (fraction 4) were obtained (Table 1) and fixed in 70% ethanol. The latter fraction also contained multinucleate round spermatids, which after treatment with pepsin dissociated into single nuclei.

**Table 1** Percentage cellular composition of cell fractions for pulse cytophotometric analysis

|                       | Total testicular suspension (%) | Spermatid fraction (%) | Pachytene fraction (%) |
|-----------------------|---------------------------------|------------------------|------------------------|
| Elongated spermatids  | 36                              | 20                     | 6                      |
| Round spermatids      | 26                              | 60                     | 27                     |
| Primary spermatocytes | 18                              | 14                     | 58                     |
| Other cells           | 20                              | 5                      | 10                     |

Cellular composition of the fractions was determined by fluorescence microscopy.

**Table 2** Calculation of coefficients of variation (CVs)

| Cell fraction          | Animal no. | CVs of peaks (%)                 |                                |          |          |
|------------------------|------------|----------------------------------|--------------------------------|----------|----------|
|                        |            | 1C                               | 1C                             | 4C       |          |
|                        |            | (2 separate peaks)<br><i>a</i> * | (combined peaks)<br><i>b</i> † | <i>a</i> | <i>b</i> |
| Total testis           | 4          | 1.0%                             | 3.5%                           | —        | —        |
| Spermatid              | 1          | 0.9%                             | 2.6%                           | —        | —        |
|                        | 2          | 1.2%                             | 2.5%                           | —        | —        |
|                        | 3          | 1.2%                             | 2.6%                           | —        | —        |
| Pachytene spermatocyte | 1          | ‡                                | 2.7%                           | 1.3%     | 2.0%     |
|                        | 2          | 1.4%                             | 2.7%                           | 0.8%     | 1.1%     |
|                        | 3          | ‡                                | 2.7%                           | 1.0%     | 1.1%     |

\* Method *a*: CV of gaussian curve fit to peaks of DNA histograms. First, the positions of the means were found by fitting second order polynomials to the top six points of each peak. The variance was estimated from the distance from the mean to a point determined by linear interpolation equal to 0.606 of the maximum height. The area of the gaussian curve was computed by the value which gave the best least squares fit when weighted by the inverse of the number of cells at each element.

† Method *b*: Coefficient of variation computed by  $CV = \mu^{-1} [\sum_i y_i (x_i - \mu)^2 / \sum_i (y_i - 1)]^{1/2}$  where  $x_i$  is the channel number,  $y_i$  is the number of counts in the channel,  $\mu$  is the mean channel, and the sum is over a range of channels from  $\mu(1 - 3 CV)$  to  $\mu(1 + 3 CV)$ . As the range is dependent on the CV, an iterative procedure was used and shown to converge.

‡ Not sufficiently resolved to obtain suitable parameters for fit by two gaussians.

Proportional fluorometry of 1C, 2C and 4C mouse testis cells can be achieved by pepsin treatment of the cells, staining with ethidium bromide and mithramycin, and measurement in a pulse cytophotometer with a high numerical aperture for excitation and measurement<sup>14</sup>. The samples were treated with 0.5% pepsin (Serva) in HCl (pH 1.8) at room temperature for from 5 min to 1 h. Pepsinisation for 10–30 min seemed to give optimal results. The samples were centrifuged and suspended in a staining solution containing 25  $\mu\text{g ml}^{-1}$  ethidium bromide, 0.1 M NaCl and 0.1 M Tris buffer (pH 7.4)<sup>6</sup>. Five minutes later an equal volume of 50  $\text{mg ml}^{-1}$  mithramycin solution containing 7 mM  $\text{MgCl}_2$  and 12.5% ethanol was added. RNase was added to a final concentration of approximately 0.02% and the sample was incubated at room temperature for at least 10 min. DNA distributions of the cells were measured using an impulse cytophotometer (ICP-22, Phywe) developed by Göhde. The ICP-22 was equipped with a glass sheath flow chamber<sup>5</sup> and was coupled to a Northern Scientific multichannel analyser.

Figure 1*a* and *b* show the DNA histograms obtained from samples of total testicular cells and of round spermatids, respectively. Two peaks in the DNA histograms can be seen. The distributions were fit with two gaussian functions and the relevant parameters calculated. The difference in DNA content between the two populations was  $3.6 \pm 0.1\%$  (s.e.m.), which is consistent with the DNA content of the X- and Y-bearing spermatids. The area under the peak with lower DNA content represents  $52.8 \pm 0.8\%$  of the 1C cells. The other peak represents  $47.2 \pm 0.8\%$ . These values are statistically significantly different from 50% at  $P = 0.05$ .

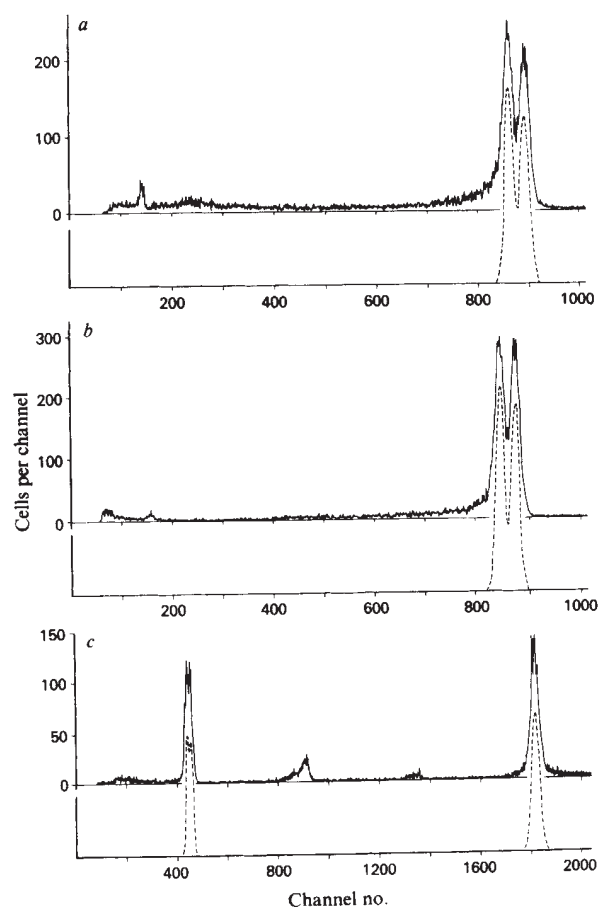
Figure 1*c* shows the DNA histogram obtained from a sample of pachytene spermatocytes. Peaks are also present in the 1C region representing round spermatids and in the 2C region representing various kinds of diploid cells. The CVs of the 4C peak of pachytene spermatocytes were as low as 0.8%, when calculated by fitting a gaussian curve. Calculation of the CV by method (*b*) (Table 2) provides a more conservative estimate. The CVs computed in the latter fashion are larger, with a minimum of 1.1%, and reflect the background counts and asymmetric broadening, whereas the gaussian fitting procedure

tends to emphasise the region of the peak. If we consider the haploid peak to be a single distribution, we obtain a value of 2.6% for its CV (method *b*). This width is larger than the values of 1.1% obtained for the 4C peak.

To determine whether there was an instrumental variation of the CV as a function of channel number or fluorescence intensity, a sample of cells (primarily lymphocytes) from mouse spleen was stained in the same manner as the testis cells. The DNA histogram revealed peaks at 2C ( $G_1$  cells) and at 4C ( $G_2$  cells and clumps of  $G_1$  cells). Both peaks had CVs of 1.5% when computed by a gaussian fit, demonstrating that there is negligible variation of CV with position. Thus, in the case of testis cells, the broader 1C peak cannot be a result of instrumental factors and must result from the heterogeneity of the haploid cells.

One possible source of such heterogeneity is the presence of spermatids at different stages of development in the cell suspensions. Elongated spermatids have a lower ability to take up stain than round spermatids<sup>15</sup>. However, the stainability increases after prolonged proteolytic digestion<sup>14</sup>. The possibility that the lower of the two peaks represents elongated spermatids is ruled out by the nearly equal number of cells in both haploid peaks, whereas the percentages of haploid cells

**Fig. 1** DNA distributions obtained from total testicular cell suspensions (*a*), and fractions of testicular cells enriched in round spermatids (*b*) and pachytene spermatocytes (*c*). Testicular cells were separated from DBA/2Tex mice (Timco) and fixed in ethanol, treated with pepsin and RNase, and stained with ethidium bromide and mithramycin. DNA distributions, measured with an impulse cytophotometer ICP-22, are shown as a solid line. Note, in spectra *a* and *b*, the 1C peak is at about channel 870, whereas in spectrum *c*, the 1C peak is at about channel 450, and the 4C peak is at about 1800. The dashed lines indicate the best fits to the peaks with gaussian distributions.





which are elongated spermatids are 70%, 25% and 17% in the total testis, round spermatid and pachytene spermatocyte samples, respectively. Thus we conclude that the two peaks represent X- and Y-bearing spermatids.

The position of the fluorescence intensity of the elongated spermatids in Fig. 1 is not known for certain. A peak at about 60% of the haploid fluorescence intensity, corresponding to elongated spermatids<sup>10,14</sup> was observed in total testis samples analyzed within one day of fixation. We propose the following explanation for the absence of this peak in Fig. 1a. After storage, elongated spermatids could increase their stainability to approach that of the round spermatids. Thus, the two peaks would represent X-bearing spermatids (both round and elongated) and Y-bearing spermatids (round and elongated). To rigorously test for the presence of elongated spermatids in these peaks, we propose to sort cells from this region using a fluorescence activated cell sorter. Furthermore, it is possible that the most highly condensed spermatids display a slightly lower fluorescence intensity, thus accounting for the broadening of the peaks to the left-hand side. It is because of this asymmetry that there seem to be more cells in the lower peak when the peaks are fitted with symmetrical distributions.

Thus, we have shown that measurements of the DNA contents of spermatogenic cells with coefficients of variation of 1% or less is possible with the pulse cytophotometer. This should permit sensitive detection of effects of mutagenic agents on testicular cells and sperm. Using prolonged proteolysis with different enzymes<sup>14</sup>, we are now attempting to resolve X- and Y-bearing mature spermatozoa on the basis of their DNA content in order to assay the success of separation of these cells.

The investigation was supported by grants from the NCI, DHEW, the NSF and American Cancer Society. We thank Betty Reid and Jill Longtin for assistance.

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Received 7 April; accepted 26 June 1978.

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## Transbilayer disposition of the phospholipid annulus surrounding a calcium transport protein

THE lipids that interact with the penetrant surfaces of membrane proteins constitute an important fraction of the lipids in biological membranes: for example, in sarcoplasmic reticulum (SR) vesicles 30 phospholipid molecules are associated with the ATP-dependent calcium transport protein (Ca-ATPase) out of a total of approximately 90 lipids per Ca-ATPase. The lipids in this fraction determine the enzymatic activity of the Ca-ATPase<sup>1,2</sup> and they show different physical properties from the remainder of the lipid bilayer<sup>2,3</sup>; lipids which do not support ATPase activity can be excluded from this fraction<sup>4</sup>. Similar behaviour has been inferred for the lipids associated with other membrane proteins including rhodopsin<sup>5</sup>, cytochrome oxidase<sup>6</sup> and glycophorin<sup>7</sup>. These lipids were thought to exist as a single bilayer shell or 'annulus' around the protein although no evidence was available to indicate the precise topographical arrangement of the lipids. We describe here a new approach to the identification of this lipid annulus which has allowed us to determine the number of annulus lipids that surround the Ca-ATPase in each monolayer.

Phospholipase D is an enzyme which removes the choline headgroup from phosphatidylcholine to yield phosphatidic acid, and which shows maximal activity in the presence of 40 mM CaCl<sub>2</sub> at pH 5.6. The digestion of dioleoyl-phosphatidylcholine (DOPC) which has been radiolabelled with tritium in the choline moiety<sup>8</sup> was followed by observing the disappearance of <sup>3</sup>H-DOPC from chloroform-methanol extracts<sup>9</sup> of aliquots of the incubation mixture. When sonicated liposomes consisting of <sup>3</sup>H-DOPC were exposed to digestion by phospholipase D, 59% of the lipids were digested, corresponding to the outer monolayer only of the liposomes (see also ref. 10); when the liposomes were disrupted by the detergent potassium cholate (2.0 mg ml<sup>-1</sup>) all of the lipid (>98%) was accessible for digestion.

More than 90% of the endogenous lipids surrounding the Ca-ATPase were replaced by <sup>3</sup>H-DOPC using the lipid substitution technique of Warren *et al.*<sup>11,12</sup>. These complexes, which consist of unsealed membrane fragments which are unable to

**Table 1** The number of phospholipids associated with the Ca-ATPase which are inaccessible to digestion by phospholipase D

| Lipid | Total lipids per Ca-ATPase | Undigested lipids per Ca-ATPase |
|-------|----------------------------|---------------------------------|
| DOPC  | 90.4                       | 29.8                            |
|       | 72.4                       | 30.4                            |
|       | 70.7                       | 28.3                            |
|       | 68.4                       | 33.4                            |
|       | 50.1                       | 31.3                            |
|       | 49.5                       | 31.2                            |
| DPPC  | 35.5                       | 27.5                            |
|       | 50.9                       | 35.3                            |
| DMPC  | 47.6                       | 32.4                            |

Complexes of Ca-ATPase with synthetic lipids were prepared and incubated with phospholipase D as described in Fig. 1. Different lipid:protein ratios were obtained by adjusting the ratio of lipid and cholate in the lipid substitution procedure<sup>2,11</sup>; complexes with DPPC and DMPC were labelled with <sup>3</sup>H-DPPC and <sup>3</sup>H-DMPC respectively. Complexes were assayed for protein by the biuret method and for lipid content by gas-liquid chromatography and radioactivity<sup>11</sup>; the added synthetic lipid comprised at least 90% of the total lipid in the complexes. The initial stoichiometries were calculated using a value for the Ca-ATPase molecular weight of 115,000 (ref. 11) and the number of undigested lipids from the plateau level of chloroform-extractable radioactivity in phospholipase D incubations.

accumulate calcium, were subjected to digestion by phospholipase D and it was found that not all of the lipid was available for digestion (Fig. 1); the amount of lipid which was digested did not change when a similar experiment was performed with potassium cholate in the incubation mixture. When the experiment was repeated using Ca-ATPase preparations which contained different initial proportions of DOPC and protein (see Table 1), the pool of lipid inaccessible to digestion always amounted to approximately 30 mol phospholipid per mol Ca-ATPase. In experiments using two other synthetic phospholipids, dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC), similar behaviour was observed (Table 1). Irrespective of the nature of the phospholipid molecule and the number originally associated with each molecule of Ca-ATPase, approximately 30 mol of phospholipid per mol Ca-ATPase are always inaccessible to attack by phospholipase D.

We suggest that this pool of undigested phospholipid corresponds to the 30 lipids in the annulus surrounding the Ca-ATPase which has been characterised by other biochemical and physical techniques (refs 1, 2 and C. Montecucco, unpublished results).

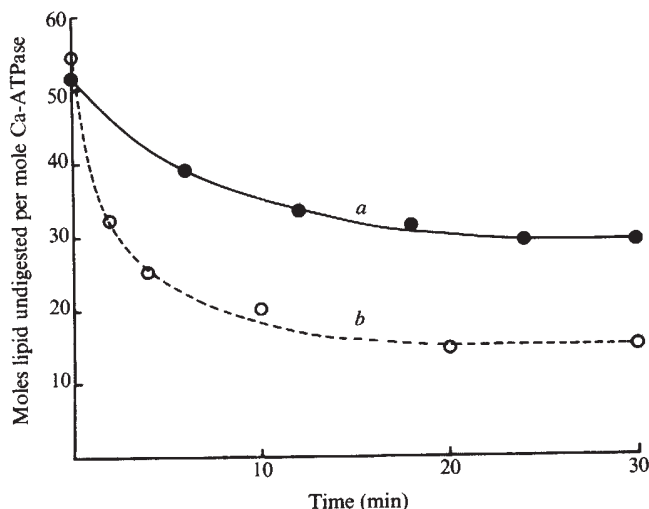
It is reasonable to suggest that the annulus lipids would remain undigested because they interact closely with the Ca-ATPase protein and hence are inaccessible to the lipase. It is not immediately clear, however, that the annulus lipids would remain next to the protein and not exchange with phosphatidic acid in the bilayer outside of the annulus; lipids in the annulus

exchange with lipids in the bilayer rapidly in relation to the time course of these experiments. The digestion with phospholipase D was carried out in aqueous suspensions in the presence of 40 mM  $\text{CaCl}_2$ . Divalent cations ( $\text{Ca}^{2+}$ ) will complex the digestion product, phosphatidic acid, forming a separate rigid phase<sup>13</sup>, and this will prevent the exchange of phosphatidic acid with the inaccessible phosphatidylcholine in the lipid annulus. That  $\text{Ca}^{2+}$  was responsible for inhibiting the exchange of phospholipids was shown in an experiment in which a Ca-ATPase complex was digested to the limiting level (after which no further digestion occurs during an overnight incubation) and washed twice in buffer containing 10 mM EGTA. On subsequent incubation with phospholipase D further digestion was found to occur, showing that the pools of undigested phosphatidylcholine and phosphatidic acid do mix in the absence of calcium.

The preparations used for the above experiments consisted of unsealed membrane fragments where the whole bilayer was exposed to attack by phospholipase D. If, however, sealed vesicles are used phospholipase D will only attack the external monolayer, and by labelling the outer monolayer of sealed, right-side out sarcoplasmic reticulum vesicles with  $^3\text{H}$ -DOPC we have been able to use phospholipase D to examine the disposition of the annulus lipids between the two halves of the bilayer.

A sealed right-side out preparation of vesicles was made by incubating SR vesicles with ATP and  $\text{CaCl}_2$  in the presence of 5 mM potassium oxalate (see Table 2 legend) so that within sealed vesicles calcium oxalate was precipitated. The dense calcium oxalate-containing vesicles were separated from leaky vesicles and inside-out vesicles by sucrose density centrifugation. The precipitated calcium was then removed from the

**Fig. 1** Time course of phospholipase D digestion of the lipids surrounding the Ca-ATPase. *a*, both sides of the bilayer for purified Ca-ATPase in a bilayer consisting of  $^3\text{H}$ -DOPC. *b*, outer side of the bilayer for sealed SR vesicles asymmetrically labelled with  $^3\text{H}$ -DOPC using a phospholipid exchange protein. The lipids surrounding the purified Ca-ATPase<sup>11</sup> were replaced by DOPC containing choline-labelled  $^3\text{H}$ -DOPC as described previously<sup>11,12</sup>; sealed SR vesicles were prepared and labelled as described in Table 2. Samples (0.5 mg) were incubated at room temperature in a volume of 1.0 ml containing 40 mM  $\text{CaCl}_2$ , 300 mM acetate buffer pH 5.6 and 3 mg ml<sup>-1</sup> phospholipase D (from Boehringer or prepared as in ref. 8). Aliquots of 0.1 ml were sampled over 30-min into 2 ml  $\text{CHCl}_3:\text{CH}_3\text{OH}$  (2:1)<sup>9</sup> in glass vials and vortexed to form a single phase. To each vial was added 0.5 ml of 5% choline chloride and after vortexing and allowing the phases to separate 1.0 ml of the lower ( $\text{CHCl}_3$ ) phase was transferred to a scintillation vial and dried under a stream of air before addition of scintillation fluid and counting. In control incubations to which no phospholipase D was added there was no change in the chloroform-extracted radioactivity. In a separate experiment (not shown) the chloroform extracts were examined by thin-layer chromatography and it was shown that the disappearance of radioactivity corresponded to the appearance of phosphatidic acid and a reduction in the amount of phosphatidylcholine.



**Table 2** The number of phospholipids in the outer monolayer of SR membranes which are inaccessible to digestion by phospholipase D

| Total lipids per Ca-ATPase in outer leaflet | Undigested lipids per Ca-ATPase |
|---------------------------------------------|---------------------------------|
| 68.3                                        | 14.8                            |
| 54.5                                        | 15.0                            |
| 35.3                                        | 14.9                            |

To SR (30 mg protein) in 30 ml buffer containing 5 mM potassium oxalate, 5 mM Mg.ATP, 100 mM KCl, 10 mM histidine buffer pH 7.3 was added, over 30 min, 5 ml of 80 mM Mg.ATP, 160 mM  $\text{CaCl}_2$  at room temperature. This was centrifuged at 65,000g for 60 min at 20°C on a 40%–60%–80% discontinuous sucrose gradient. The band which penetrated the 60% sucrose layer was suspended in 22 ml 10 mM  $\text{MgSO}_4$ , 1 mM Mg.ADP, 3 mM EGTA, 125 mM sucrose, 0.5 M KCl, 50 mM potassium phosphate buffer pH 8.0 to which was added 0.5 mg (247 units) hexokinase (Sigma) and 0.4 mmol glucose to form an ADP regenerating system. The SR was washed twice into 250 mM sucrose, 1M KCl, 50 mM phosphate pH 8.0 and then centrifuged at 65,000g for 60 min at 20°C on a discontinuous sucrose gradient as before. The band which failed to penetrate the 60% sucrose layer (calcium oxalate-depleted vesicles) was taken and 3 mg was incubated at 37°C with 1.0 mg sonicated  $^3\text{H}$ -DOPC and 1.0 mg (~240 units<sup>14</sup>) phospholipid exchange protein (prepared from beef heart<sup>14</sup>) in 250 mM sucrose, 1 mM EDTA, 50 mM Tris-Cl pH 7.4. The mixture was centrifuged at 250,000g for 90 min into 15% sucrose with a 50% sucrose pad and the band at the 15%–50% interface was washed twice into 1M KCl, 50 mM phosphate buffer pH 8.0. Over 98% of the  $^3\text{H}$ -DOPC radioactivity could be exchanged out of the SR on a subsequent incubation with excess unlabelled DOPC and the phospholipid exchange protein; less than 4% of the lipid incorporated was due to fusion in control experiments using sonicated DOPC containing traces of  $^{14}\text{C}$ -triolein (Amersham) which is not exchanged by the phospholipid exchange protein. The labelled SR vesicles were treated with phospholipase D as described in the legend to Fig. 1, and in order to calculate the stoichiometries the percentage of SR protein which is Ca-ATPase (around 95% for most sealed SR preparations) was estimated by SDS-polyacrylamide gel electrophoresis. The SR vesicles remained sealed at the end of the procedure as demonstrated by their capacity for ATP-dependent calcium uptake.



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**Table 1** Nearest-neighbour matrix for site  $k$ 

|             | 1   | 2      | $\nu_{k+1} = 3$ | 4   | 5      |
|-------------|-----|--------|-----------------|-----|--------|
| $\nu_k = 1$ | 1   | 1      | 1               | 0   | 1      |
| 2           | $x$ | $u_1x$ | $u_2x$          | 0   | $u_sx$ |
| 3           | 0   | 0      | 0               | $y$ | 0      |
| 4           | $y$ | $u_2y$ | $y$             | 0   | $y$    |
| 5           | $z$ | $u_sz$ | $z$             | 0   | $z$    |

'interaction': for example, if site  $k$  is in state 1, it is impossible for site  $k+1$  to be in state 4; or, if site  $k$  is in state 3, site  $k+1$  cannot be in state 5, and so on. In a formal way, there are infinite repulsions ( $w = \infty$ ) between such neighbouring states.

The next step<sup>2</sup> is to fill in the nearest-neighbour matrix for site  $k$ , as shown in Table 1, where  $\nu_k$  refers to the state of site  $k$ . The entries are the weights for site  $k$  [ $x \equiv K_1c$ ,  $y \equiv (K_2c)^{1/2}$ ,  $z \equiv K_sc_s$ ], multiplied by factors  $u_i = \exp(-w_i/kT)$  for interactions between sites  $k$  and  $k+1$ . The 'infinite repulsions' referred to above produce the zero entries.

What is needed is the largest eigenvalue  $\gamma_{\max}$  of the matrix in Table 1, that is, the largest root  $\gamma_{\max}$  of the characteristic equation

$$\begin{vmatrix} 1-\gamma & 1 & \dots \\ x & u_1x-\gamma & \\ \vdots & & \end{vmatrix} = 0 \quad (1)$$

The grand partition function of the system<sup>2,6</sup> is then given by  $\ln \Xi = M \ln \gamma_{\max}$ . Finally,

$$\text{HMM: } \theta = \bar{N}/M = c \partial \ln \gamma_{\max} / \partial c \quad (2)$$

$$\text{S1: } \theta_s = \bar{N}_s/M = c_s \partial \ln \gamma_{\max} / \partial c_s$$

Carrying out this program is awkward or requires numerical methods if equation (1) is of higher degree than quadratic. To convert equation (1) into a quadratic, we take  $u_1 = u_2 = u_s = 1$  (that is, no interactions). What we have left in our binding model is the competition of S1 binding and of two kinds of HMM binding for the same sites.

With  $u_1 = u_2 = u_s = 1$ , we find from equation (1) (because three roots are  $\gamma = 0$ )

$$2\gamma_{\max} = 1 + x + z + [(1+x+z)^2 + 4y^2]^{1/2} \quad (3)$$

Using equation (2), we then deduce

$$\theta = \frac{(1+K_1c+K_sc_s+\sqrt{K_1c+2K_2c})(1+K_1c+K_sc_s+\sqrt{K_1c+2K_2c})}{(1+K_1c+K_sc_s+\sqrt{K_1c+2K_2c})} \quad (4)$$

$$\theta_s = K_sc_s/\sqrt{K_1c+2K_2c} \quad (5)$$

where

$$\sqrt{K_1c+2K_2c} = [(1+K_1c+K_sc_s)^2 + 4K_2c]^{1/2}$$

These are the desired binding isotherms for the two ligand species. Note that equation (4) can also be written as

$$\begin{aligned} \theta &= \theta_1 + \theta_2, & \theta_1 &= K_1c/\sqrt{K_1c+2K_2c} \\ \theta_2 &= 2K_2c/(1+K_1c+K_sc_s+\sqrt{K_1c+2K_2c}) \end{aligned} \quad (6)$$

Thus the expressions for  $\theta$  and  $\theta_s$  are consistent with equations (32) and (33) of ref. 2.

There are several special cases of interest. (a) If  $K_2 = 0$ , there is simple competition between two monovalent species:

$$\theta = K_1c/(1+K_1c+K_sc_s), \quad \theta_s = K_sc_s/(1+K_1c+K_sc_s) \quad (7)$$

(b) If  $K_1 = K_s = 0$ , we have the pure 'parking' problem for a single divalent species:

$$\theta = 2K_2c/[1+(1+4K_2c)^{1/2}](1+4K_2c)^{1/2} \quad (8)$$

This gives  $\theta \rightarrow K_2c$  for  $c \rightarrow 0$  and  $\theta \rightarrow 1/2$  for  $c \rightarrow \infty$ .

(c)  $K_1 = 0$  in equations (4) and (5) applies if binding as in state 2, Fig. 1 is unimportant.

(d)  $c_s = 0$  in equation (4) applies to the binding of HMM in the absence of S1. In this case, if  $K_2 \gg K_1$ , two-headed binding (states 3 and 4 in Fig. 1) will predominate when  $c$  is small, but single-headed binding (state 2 in Fig. 1) will eventually take over as  $c \rightarrow \infty$  (that is,  $\theta \rightarrow 1$ , not  $\frac{1}{2}$ ).

For comparison, consider binding on an ensemble of separated pairs of sites ( $M = 2$  rather than  $M = \infty$ ). Of course it would be simple to put interactions into this model<sup>6</sup>. The grand partition function for one pair of sites is<sup>6</sup>

$$\xi = 1 + 2K_1c + K_1^2c^2 + 2K_sc_s + K_s^2c_s^2 + 2K_1cK_sc_s + K_2c \quad (9)$$

There are ten terms here, each representing a possible state of binding of the two sites. Furthermore, each term is proportional to the equilibrium probability of the corresponding state. Thus we can write immediately, on inspection of equation (9),

$$\theta = \bar{N}/2 = (K_1c + K_1^2c^2 + K_1cK_sc_s + \frac{1}{2}K_2c)/\xi \quad (10)$$

$$\theta_s = \bar{N}_s/2 = (K_sc_s + K_s^2c_s^2 + K_1cK_sc_s)/\xi$$

In the special case  $K_1 = 0$ ,

$$\begin{aligned} \theta &= \frac{1}{2}K_2c/[(1+K_sc_s)^2 + K_2c] \\ \theta_s &= K_sc_s/(1+K_sc_s)^2 + K_2c \end{aligned} \quad (11)$$

An interesting question (posed by E. Eisenberg) is: in the  $K_1 = 0$  case, with both  $c$  and  $c_s$  large (virtual saturation of the sites), what is the relation between  $c$  and  $c_s$  such that as many sites will be occupied by HMM as by S1? In the  $M = 2$  case, the dominant terms in equation (9) (with  $K_1 = 0$ ) are  $K_s^2c_s^2$  and  $K_2c$ . Hence the required condition is simply  $K_s^2c_s^2 = K_2c$ . This also follows, of course, from equations (11). The answer to the same question for the  $M \rightarrow \infty$  system (with  $K_1 = 0$ ) can be seen from equations (4) and (5) to be  $(3/4)K_s^2c_s^2 = K_2c$ . That is, with  $K_s$ ,  $K_2$ , and  $c_s$  held constant, a somewhat lower concentration  $c$  of HMM suffices for the  $M \rightarrow \infty$  model.

In future work, it is planned to apply these equations to experimental data and to discuss the theoretical basis for the observed relative magnitudes of  $K_s$ ,  $K_1$ , and  $K_2$ .

I thank Dr Evan Eisenberg for reviving my interest in this kind of binding problem.

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Received 20 March; accepted 23 June 1978.

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# reviews

## Highlighting risks of nuclear power

J. H. Fremlin

*The Silent Bomb: A Guide to the Nuclear Energy Controversy.* Edited by P. Faulkener. Pp.382. (Random House: New York; Friends of the Earth International: San Francisco, New York, London and Paris, 1978.) \$10.95.

THIS could have been a most useful book. However carefully engineers in the nuclear industry may examine safety precautions, one can never be sure that they will think of everything. It is inherently more likely that informed opponents of nuclear power will locate unconventional and unlikely routes to accidents, and any suggestions of such unconsidered risks from outsiders should merit very careful consideration by reasonable people in the industry concerned as well as by the interested general public.

The book begins with a powerful foreword by the versatile Dr Paul Ehrlich, who some years ago was exceedingly concerned about the dangers resulting from too many human beings on this planet, but who now seems to have convinced himself that there is an even bigger risk of having too few human beings on this planet. It will be interesting to see whether in another ten years he will be demonstrating the same level of apocalyptic concern about the increase in the carbon dioxide level in the atmosphere due to the burning of fossil fuels.

Unfortunately the prevalence of absurd inaccuracies and the near-hysterical tone of much of the book make it exceedingly unlikely that anyone, other than convinced supporters of the anti-nuclear movement, will take any notice of it at all.

On the outside of the cover, before we even open the book, it is stated that: "A nuclear plant accident could kill 50,000 people, damage \$17 billion worth of property over a 600,000 square mile area, inflict an untold number of injuries and genetic mutations—it could happen today!". The area concerned is about eleven times the area of Great Britain and not even a military weapon has yet been produced which could inflict damage to property over anything like this area. A hydrogen bomb of 100 megatonnes equivalent exploded on the ground would certainly give lethal

doses to a lot of people as far away as some hundreds of miles downwind but it is really difficult to see how it could affect property over a radius of 450 miles. Nuclear power plants may be subject to melt-downs and in some cases to steam or chemical explosions or fires, but they are not subject to nuclear explosions even at the A-bomb level.

Similarly, on p281, Dale G. Bridenbaugh, Richard B. Hubbard and Gregory C. Minor state their belief that nuclear fission power is so dangerous that it threatens the very existence of life on this planet. Some millions of rads in an hour or so over the entire world? This planet carries some very resistant organisms. To take just one technical howler from the text, someone genuinely trying to learn how a reactor works is going to be very much confused by the statement (pp80-81):

"A fission chain reaction is self-sustaining when the number of neutrons released in a given time equals or exceeds the number of neutrons that are absorbed by non-fissioning material or that escape from the system".

Little credit for any of these statements seems to be due to the dozens of people cited by name as having helped with several drafts of the book.

The chapters of which I know nothing, such as those concerned with the American economics and politics of nuclear energy, are extremely convincing. I find it only too easy to believe that big industrial firms will use every possible method, including bribery and large scale propaganda, to forward their own interests. The only thing that I find suspicious is that no consideration should have been given to the probability that those big organisations selling coal or oil which have not covered themselves by investment in the nuclear industry, are behaving in exactly the same way. I do not, of course, believe that Messrs Bridenbaugh, Hubbard or Minor were paid by the coal industry to abandon their positions and salaries in the nuclear industry. Why should it do so if they were willing to do its propaganda free?

There are, however, plenty of other ways in which a big established industry which sees itself threatened by a smaller new one can encourage the

right attitude in the public; whether by direct contributions to senators as the nuclear industry is convincingly reported as making; by publishing technical literature on the dangers of nuclear power which omits to mention the dangers of other sources; or by planting individuals in likely areas to lead independent protests against the building of nuclear power plants. In Britain the number of people who die as a result of air pollution by the burning of fossil fuel must run into thousands per year, apart from the couple of coal miners who die every day from accident or pneumoconiosis, and although it is to be hoped that both of these figures will improve with time, it is not easy to see how they can reduce to anywhere near the number, two or three per year, who are likely to die as the result of the normal operations of our nuclear industry.

The sections of the book describing a number of ways in which pressurised water reactors may suffer disastrous breakdowns are valuable, although again I would expect them to carry more actual weight if some estimates were made of the likelihood of the several necessary factors occurring at once. The impression left on reading the section concerned is that large numbers of such accidents are imminent; but the 1,100 reactor-years which have so far occurred, without a single person having been killed by the radiations from a nuclear power station, make it seem highly unlikely that such major accidents—which would have to occur about once a year to match the death rate due to the burning of coal—could have anything like this probability.

The clear, and so far as I know accurate, description of the incidents which have occurred form a somewhat ludicrous anti-climax to the discussion of the accidents which might be expected to occur. To describe the accident at the Enrico Fermi reactor under the title "We Almost Lost Detroit" is such an exaggeration of what could have happened with the type of plant concerned that one tends to discount the whole of the chapter. This is a pity, because many of the criticisms of the safety procedures on pressurised water reactors are fair; such reactors would certainly not have

been passed for construction by our own Nuclear Installations Inspectorate (NII). Indeed much of the book, if written in a more convincing tone, would form a very powerful argument for the establishment in the US of just such an independent body as our NII which would have to accept and pass the full designs before construction was permitted. It may be that the lack of success of British industry in selling reactors abroad is due, not to the inefficiency often imputed, but to the fact that the installation of desirable safeguards has made them so much more expensive than American re-

actors without such safeguards.

To summarise, quite a lot of the book is useful so long as one reads it not as a guide to the nuclear energy controversy, which it certainly is not, but as a highlighting of the risks, from the small but probable to the enormous but highly improbable, and as a very revealing statement of the point of view of the opponents of nuclear power. □

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## Supernovae astronomy

*The New Astronomy.* By P. Murdin and L. Murdin. Pp. 215. (Reference International: London; Thomas Y. Crowell: New York, 1978.) £5.95; \$12.95.

ANYONE who has lectured to an amateur astronomical society, or a school class, knows that it is the question from the youngest member of the audience, from the least expert of the assembled, that is usually the most difficult to answer. I'll never forget my very first lecture, which revealed me as the green-horn I was. After expounding on the glories of solar physics the first questioner, a seven year old, simply asked me "Why was the Sun called Sun?". Exit lecturer in confusion.

In general it is the hard, new, incomplete and speculative branches of astronomy that interest the lay students. Subjects that are well known and explicable are dismissed as 'boring'. Just mention blackholes, supernovae, curved space, pulsars and quasars and you can literally see the ears prick up and the whiskers twitch.

The Murdins' book is for these people. It reads like a good novel; it is hard to put down and can be gobbled up in one go. The hero of the piece is the supernova of AD 1054 and its present day association, the Crab Nebula, Messier 1 in Taurus. As such the title of the book is a touch misleading. It should really be *Supernovae Astronomy*.

Paul and Lesley Murdin take to their hearts Geoffrey Burbidge's aphorism "contemporary astronomy can be divided into the study of the Crab Nebula and the study of everything else". An exaggeration obviously, but supernovae, their gas remnants, pulsars, blackholes, radio, X-ray and neutrino emissions and their roles as cosmic ray

sources, birth places of the heavy elements and possible triggers for planetary formation, do hold a central position in modern astronomy.

The mysteries of supernovae are revealed chronologically, the authors starting with the Chinese descriptions of the guest star which was first noted on 4 July, 1054. The Renaissance supernovae of 1574 and 1604 are discussed in detail, special emphasis being made of the way in which these two new stars shattered the crystal spheres of Aristotelean thought. In the old astronomy celestial perfection started beyond the Moon and the firmament of stars was immutable. This was disproved by Tycho Brahe's measurements of the parallax of his nova, a measurement which placed it at least ten times further away than the Moon.

## Paper and thin-layer chromatography

*Laboratory Handbook of Paper and Thin-Layer Chromatography.* By J. Gasparic and J. Churacek. Pp. 362. (Wiley: New York and Chichester, 1978). £18.

THE blurb on the dustcover of this book is most revealing. First, it tells us that "exhaustive references include the latest experience and results from East European sources.". Yet 95% of the references are pre-1974 and 90% pre-1970, which will fill many of us with envy at the thought of the very long holidays they must have. Second, it tells us that the two authors have already written "five books on paper and thin layer chromatography" and one can well believe it from a viewing of this, their sixth, work.

Part 1 comprises seventyseven pages and describes the principles, techniques and uses adequately. Part 2, the rest of the book, is devoted to the applications

The fact that the Galaxy should have a bright supernova every 200 years but that none has occurred since the invention of the telescope, is bemoaned. Hartwig's discovery of SN Andromedae in 1885 and its role in the Great Debate is mentioned. Then follows an account of how the veils of mystery of the Crab Nebula were peeled off one by one: Winlock and Pickering (1868) found that it was gaseous; Slipher (1913) found that it was expanding; Hubble (1928) connected it with the supernova of 1054; Baade (1945) noticed that the expansion was accelerating; Bolton and Stanley (1948) identified the radio star Taurus A as the Crab Nebula; X-ray astronomers at the Naval Research Laboratory, Washington (1963), discovered the Crab to be an X-ray source; Hewish and Bell (1968) discovered pulsars; and Staelin and Reifenstein searched the Crab and found one there; and Cooke, Disney and Taylor found light flashes emanating from the centre. It is a long list and one which will surely continue. The book ends with chapters on neutron stars, neutrino astronomy, element creation, cosmic rays and blackholes.

*The New Astronomy* by Paul and Lesley Murdin is a feast for the amateur astronomer and can be highly recommended.

David W. Hughes

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which cover the whole range of inorganic, organic and biological chemicals. However, about half of this section is devoted to tables of  $R_f$  values, solvent and reagent compositions drawn entirely from the literature in what seems to be a fairly uncritical manner. A more careful look at the sections which particularly interest me shows that a total of two pages are devoted to amino acids, one to ketoacids, five to sugars and seven to lipids, and the whole subject of radioactive compounds is covered in less than three pages.

The professional analyst will certainly know far more about the subject than what is included in this work, whereas the student will need a much more critical and explanatory book. At all levels, there are better and far less expensive books available which I would recommend in preference to this £18 text.

Ivor Smith

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## Molecular immunology

*Immunochemistry: An Advanced Textbook.* Edited by L. E. Glynn and M. W. Steward. Pp. 628. (Wiley: New York and Chichester, 1977.) £24.

RECENT years have seen the publication of several excellent advanced texts in cellular immunology and in practical methods in immunology. The immunochemist, or indeed anyone interested in reading about general molecular immunology at a level beyond undergraduate textbooks, has not been so well served and has had to rely on out-dated books or on isolated reviews. This volume is therefore a particularly timely and welcome publication. It is a collection of reviews covering the structure, function, genetics and biosynthesis of antibodies, their reactions with antigens (in general and in certain selected cases) and the complement system.

I particularly appreciated the chapter by Stanworth on immunoglobulino-pathies. Easily understandable to the non-clinician, this chapter presents the clinical background to multiple myeloma and other immunological disorders that underlies so much of our knowledge of antibody structure and function but is often absent from the non-clinical literature. A short chapter on amyloid (Pras and Gafni) also bridges the clinical and non-clinical aspects of the subject. The chapters on three-dimensional immunoglobulin structure (Feinstein and Beale; Richards *et al.*) are extremely good non-mathematical accounts of crystallographic and physicochemical techniques and results. Thermodynamics and kinetics of antibody-antigen reactions are also presented in a very readable explanation of the factors relating to antibody affinity and activity (Steward).

Williamson, in a chapter on the origin of antibody diversity discusses evidence relating to, and some models that have been proposed to account for the enormous diversity of antibodies. He concludes that a germ-line basis for antibody diversity best explains the available data. It is unfortunate that this view is not balanced by an equally forceful presentation of the case for the somatic generation of antibody diversity elsewhere in the book.

The chapter on complement (Fearon and Austen) is a straightforward account of the components and reactions of the complement systems. I should have liked to have seen more discussion of the genetic association between certain complement components and the major histocompatibility system. Space prevents me from men-

tioning all sixteen chapters, but one other section that I found very informative was that on structure-function relationships of immunoglobulins (Turner).

The length, degree of complexity and flavour of each chapter varies widely according to the specialist authors chosen to contribute in their field. The style adopted ranges from a straightforward summary of data in some chapters to a more speculative, and even controversial, discussion in others. However, each chapter is followed by an extensive list of references (up to 1975 or early 1976), allowing the more serious student to sample the original literature. There is also what seems to be a fairly comprehensive index, though my only genuine attempt to use it—to find the reference to Forssmann, (or is it Forsmann, as both spellings are used in this book) antibody, given as p383—was unsuccessful. Cross-referencing between chapters could also have been made easier by indicating the chapter number on each page (or at the beginning of each section).

My main criticism of the book, how-

ever, arises from its claim to be a textbook. The variety of authors' styles—one of the attractions of the volume as a collection of reviews—is not well suited to a textbook. Nor is the overlap of data (and even photographs) between chapters; and the numerous minor errors that, in the interests of topicality, one might excuse in a review as a consequence of hurried proof-reading, should not be acceptable in a textbook.

But whatever one calls it, I enjoyed reading this volume enormously. My copy is already well-thumbed, but I shall enjoy re-reading many of the chapters and will also frequently refer to it for information. It is most unfortunate that the publishers seem to be resigned to library sales only—at £24 I cannot see the book becoming an immunologist's *vade mecum*, but it would nevertheless be a useful addition to any immunology library.

David Secher

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## Epithelial physiology and ion transport processes

*Membrane Transport Processes.* Vol. 1. Edited by J. F. Hoffman. Pp.476. Vol. 2. Edited by Daniel C. Tosteson, Yu. V. Ovchinnikov and Ramon Latorre. Pp.451. (Raven: New York, 1978.) \$42 each volume.

It was with some trepidation that I agreed to review these two volumes. All too often these days the ephemera of meetings are presented to us encased in the respectability of hard covers and my hope was that these volumes would not prove to be just another example of this publishers' ploy. As I began to read, it became clear that at least for volume 1 my fears were groundless.

The first volume arose out of a small meeting held in memory of Peter Curran. To the papers given on that occasion, others have been added to produce a very useful survey of epithelial physiology and closely related topics. Almost without exception the chapters are authoritative and scholarly contributions of lasting value—a fitting tribute to Peter Curran, whose ideas and work in this field were so seminal. The names of most of the best known American epitheliologists are to be found amongst the contributors; but it is perhaps a pity that the rest of the world is rather poorly rep-

resented. One might have hoped for a chapter on the recent exciting contributions of electron microprobe X-ray analysis, and it is unfortunate that the editor has allowed Diamond to dismiss the work of Hill in a series of asides when Hill could have been invited to put his own case. I am sure that Peter Curran would have wished Hill's views—right or wrong—to have been given a less biased hearing.

These criticisms are, however, of a very minor nature and should not be allowed to detract from an excellent volume. The editor and authors are to be congratulated on producing a valuable work of scholarship that will be much used in years to come.

Try as I must, I cannot say the same about volume 2, which covers a very broad range of topics concerned with the selective transport of ions across biological membranes. Here the main selling point seems to be a number of contributions from our Russian colleagues. The volume is very mixed, with some excellent articles—for instance, Kostyuk and Krishtal on ionic and gating mechanisms in molluscan neurones; but also some very poor ones. It lacks the consistent scholarly solidarity of volume 1 and it is difficult to understand for what purpose this particular *pot pourri* of papers was put together. Regrettably, I cannot place this volume in even the same league as its sister.

P. F. Baker

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## Molecular mechanisms of toxin damage

*Specificity and Action of Animal, Bacterial and Plant Toxins.* (Receptors and Recognition, Series B, Vol. 1.) Edited by P. Cuatrecasas. Pp. 345. (Chapman and Hall: London, 1978.) £15.

THAT some bacteria can kill without grossly invading their animal or human hosts has impressed and intrigued workers for half a century. The emergence of satisfactory explanations of the molecular means by which the toxins produced by such micro-organisms wreak their damage is described in this book. It has been a long and tortuous path and for some of these highly poisonous proteins one suspects that many a twist and turn lie ahead.

To start this review by talking of bacterial toxins is not to denigrate two chapters in the book, one of which by Albuquerque and Daly deals with steroid alkaloid toxins and the other by Olsnes and Pihl with abrin and ricin of plant origin, but rather to emphasise that six out of the eight chapters deal with bacterial products.

A first and striking impression on reading through the book is that of the very different levels of explanation reached for the different bacterial products. Where the excellence of molecular biological understanding can be used, as for example in the actions of cholera toxin or diphtheria toxins, satisfyingly complete pictures can be drawn. When our knowledge of molecular events involved in nerve function is tested, as in the actions of tetanus or botulinum toxin, then we have to stop short of a rounded picture at the molecular level. Undoubtedly the work on the two former toxins is among the triumphs of combined biological research and the chapters by Bennett and Cuatrecasas on cholera toxin and by R. J. Collier on diphtheria and *Pseudomonas aeruginosa* toxins are models of clarity in telling the exciting stories.

In a chapter on the large number of toxins that act on cell membranes, Alouf collects a wide range of interesting and important facts. Many years ago when  $\alpha$  toxin of *Clostridium perfringens* was first shown by Macfarlane and Knight to be a lecithinase, high hopes were felt that this example was the first of many. Indeed a number of such haemolysins have been shown to attack phospholipids but perhaps an even larger number are likely to exert

their effect by being surface-active agents whose precise mechanism of action is uncertain.

Work on the nerve toxin of botulinum is described with great clarity by L. L. Simpson. One is inclined to exclaim only at the perverseness of nature. This peripheral nerve toxin has for many years been suspected to interfere with acetylcholine action. It is surely perverse that it interferes neither with the manufacture nor with receptors for the substance, but, as is now strongly suspected, with its release.

A spirit of adventure must have inspired the inclusion of a chapter on colicins into a book on toxins. It is, however, a very apt and long-sighted action. The chapter by Holland clearly shows that investigation of colicin E3, a "toxin" for *Escherichia coli*, in many ways parallels those of the toxins for

animals, with specific receptors in the outer membrane of the bacteria, albeit protein instead of gangliosides, and subsequent penetration of the cell to disrupt a ribosomal RNA.

The book, or at least parts of it, could be read with profit by most biologists interested in molecular mechanisms, including more advanced students. It is on the whole well produced, with excellent diagrams. A little more careful proof-reading would have eliminated the rather large number of typographical errors, sometimes two or three on a page. The book is up-to-date, as far as one can expect of books, the references reaching 1976.

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## High performance liquid chromatography

*Introduction to High Performance Liquid Chromatography.* R. J. Hamilton and P. A. Sewell. Pp.183. (Chapman and Hall: London; Halsted: New York, 1978.) £8.

HIGH performance (or high pressure) liquid chromatography (HPLC) is one of main 'high growth' areas in modern instrumental chemical analysis. It is based on the practical realisation of the theoretical prediction that a spectacular increase in chromatographic column efficiency could be obtained by a reduction in the mean particle size of column packing materials to the order of 10–40  $\mu\text{m}$ . This increase could be translated into a 20- to 100-fold reduction in analysis time, or into a corresponding improvement in chromatographic separation. These benefits were obtained only by an increase in operating pressures up to 5000–6000 lb in<sup>-2</sup> and usually by a decrease in effective column load to the microgram scale. These requirements have been met by the development over the past decade of complex (and expensive) instrumentation and of a series of new, specially designed stationary phases.

These developments have been reviewed in several books, and the present volume attempts to compress the theory, practice and applications of HPLC into 172 pages. Although the coverage is reasonably complete, the attempt is only partially successful. The limitations of space have necessitated a rather terse and dogmatic style which is effective only so far

as the information conveyed is accurate. Unfortunately there are several lapses in both text and illustrations. These may be due to an imperfect acquaintance with the subject as "agarose . . . is very resistant to compressive strain" (p92); or carelessness in material, "If  $K_0 = 1$  (i.e. all molecules are excluded)" is incompatible with the preceding equation (2.66) (pp34–35); or in proof-reading, (equations 2.20 and 2.16); and on p30 " $R_s$  is proportional to  $N$ " (actually to  $N^{1/2}$ , see equation (2.55)). Some of the diagrams are also misleading: for example, Fig. 3.5 shows a pump in which the piston cannot follow the cam, and in which only the piston stem actually causes liquid movement. In Fig. 7.5 illustrating the re-cycle technique, no pump is included in the re-cycle circuit.

The most useful part of the book is chapter 4 which provides an excellent tabulated guide to the majority of high performance stationary phases now available, with comments on the packing, care and operation of HPLC columns. The relatively new technique of preparative HPLC is dealt with in chapter 7, and the concluding chapter 8 provides more than 80 examples of the applications of the method in a useful standardised format. About half of these were taken from manufacturers' literature.

Although this book provides a bird's-eye view of the whole field, it presents relatively little new material and anyone wishing to acquire a practical knowledge of HPLC would need to consult a more comprehensive and authoritative text.

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